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(54) Title: GENE THERAPY USING TARGETED VIRAL VECTORS

(57) Abstract

A general method for delivering genes to specific target cells *in vivo* is described. Enveloped viruses are genetically engineered to infect specific target cells by replacing the cell surface receptor recognition domain of viral envelope proteins with ligands that direct the binding and fusion of these viruses to specific cell surface molecules.

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GENE THERAPY USING TARGETED VIRAL VECTORS

Background of the Invention

The invention relates to gene therapy methods.

Gene therapy is an approach to treating a broad range of diseases by delivering therapeutic genes directly into the human body. Diseases that can potentially be cured by gene therapy include 1) diseases associated with the aging population such as cancer, heart disease, Alzheimer's disease, high blood pressure, atherosclerosis and arthritis; 2) viral infectious diseases such as acquired immune deficiency syndrome (AIDS) and herpes; and 3) inherited diseases such as diabetes, hemophilia, cystic fibrosis, and muscular dystrophy.

Current methods of delivery of new genetic information into cells *in vitro* include cell fusion, chromosome-mediated insertion, microcell-mediated gene transfer, liposome DNA carriers, spheroplast fusion, DNA-mediated gene transfer, microinjection, infection with recombinant RNA viruses, and infection with recombinant DNA viruses (Martin, J.C., 1984, *Mol. Cell Biochem.* 59:3-10). These techniques are not generally applicable, however, for use in animals or humans because of low efficiency, instability of introduced genes, introduction of extraneous or undesirable genetic information, and lack of target specificity.

In one particular example, a favored approach for human gene therapy involves the transplantation of genetically-altered cells into patients (Rosenberg, et al., 1988, *New Eng J Medicine* 323:570-578). This approach requires the surgical removal of cells from each patient to isolate target cells from nontarget cells. Genes are introduced into these cells via viral vectors or other means, followed by transplantation of the

genetically-altered cells back into the patient. Although this approach is useful for purposes such as enzyme replacement therapy (for example, for transplantation into a patient of cells that secrete a hormone that diseased cells can no longer secrete), transplantation strategies are less likely to be suitable for treating diseases such as cystic fibrosis or cancer, where the diseased cells themselves must be corrected. Other problems commonly encountered with this approach include technical problems, including inefficient transduction of stem cells, low expression of the transgene, and growth of cells in tissue culture which may select for cells that are predisposed to cancer. Finally, inappropriate expression of transplanted genes in nontarget cells may actually be harmful to patients.

An alternative approach to gene therapy involves the direct delivery of genes to target tissue *in situ*. Two methods for *in situ* delivery of genes have been developed: biolistic transfer and double balloon catheterization. Biolistic transfer of genes involves shooting DNA-coated platinum or gold microprojectiles directly into target tissue. Biolistic transfer has been successful in the transient expression of genes in the ear, skin and surgically-exposed liver of live mice (Johnston, S.A., 1990, *Nature* 346:776-777; Williams, R.S., et al., 1991, *Proc Natl Acad Sci USA* 88:2726-2730). Double balloon catheterization transduces genes into cells within a defined arterial wall segment. In this approach a double balloon catheter is inserted into an artery until the end of the catheter is located within the target area. Inflation of two balloons at the end of the catheter creates an enclosed space into which retrovirus or DNA-loaded liposomes are infused. This method has been successful in the transient expression of β-galactosidase genes within a defined segment of the

ileofemoral artery of pigs (Nabel E.G., et al., 1990, *Science* 249:1285-1288). Both biolistic transfer and double balloon catheterization however, although locally specific, may be nonspecific in the individual cells that they transduce within the target area, creating a problem of inappropriate gene regulation if the transgene is expressed in nontarget cells. Moreover, neither biolistic transfer nor double balloon catheterization have been shown to be effective for the treatment of tissue occupying large volumes such as lungs, muscles, tumors, or cells of the systemic circulation since the majority of the cells would be inaccessible for *in situ* gene transfer.

A third approach to gene therapy is the delivery of genes to cells *in vivo*. This approach involves the introduction of viral vectors directly into patients by injection, spray or other means. Different species of viruses are engineered to deliver genes to the cells that the viruses normally infect. Adenovirus, for example, which normally infects lung cells, has been developed as a vector to target genes to lung cells (Rosenfield, et al., 1992, *Cell* 68 143-155). Most viral vectors, however, are single purpose vectors since they can only deliver genes to certain cells. Because the target cell specificity of viral vectors is restricted to the normal tropisms of the viruses, viral vectors are generally limited in that they either infect too broad a range of cell types, or they do not infect certain types of cells at all.

Liposomes have been designed to deliver genes or drugs to specific target cells *in vivo*. By chemically conjugating antibodies or ligands to liposomes, liposomes have been targeted to specific cells. With this method, antisense *env* RNA has been delivered to human immunodeficiency virus (HIV)-infected lymphocytes using

anti-CD3-conjugated liposomes (Renneisen, K., et al., 1990, *J Biol Chem* 265:16337-16342); chloramphenicol transacetylase (CAT) genes have been delivered to $H2K^k$ positive lymphomas in $H2K^k$-negative nude mice using anti-$H2K^k$-conjugated liposomes (Wang, C. et al., 1987, *Proc Natl Acad Sci USA* 84:7851-7855); and xanthine guanine phosphoribosyltransferase (XGPRT) genes have been delivered to immunoglobulin-coated cells using staphylococcus protein A-conjugated liposomes (Machy P., et al., 1988, *Proc Natl Acad Sci USA* 85:8027-8031). The major drawback to this technology can be the expense of mass producing ligand-conjugated liposomes.

Wu et al. report a method to target naked DNA to specific cells. Asialoglycoprotein-DNA complexes are targeted to hepatocytes expressing the asialoglycoprotein receptor (Wu G.Y., et al., 1991, *Biotherapy* 3:87-95). Similar to the problem encountered with immunotoxins, however, this strategy generally limits delivery of DNA to cells expressing receptors that are capable of DNA-internalization.

Antisense DNA technology is a method for inhibiting the expression of specific genes with complementary DNA (Moffat, 1991, *Science* 253:510-511). Although antisense DNA is specific in the genes that it affects, it is nonspecific in the types of cells that it gets into. This can create problems *in vivo* because it is desirable that endogenous genes in normal cells remain unaffected by antisense DNA (e.g., protooncogenes). Moreover, the cost of manufacturing and administering antisense DNA may be high because the phosphate moieties of antisense DNA must be chemically modified to allow passage through the plasma membrane, a process which entails expensive organic chemistry. Millimolar concentrations of antisense DNA are required to be effective, posing problems of potential toxicity *in vivo*.

Human gene therapy is therefore limited by the available technology for gene delivery. Transplantation strategies, which require surgery, limit gene therapy to an expensive service industry for a small number of diseases. Targeting of genes *in situ* through local transduction is generally not precise enough. Viral vectors limit the delivery of genes *in vivo* to cells that the viruses normally infect. Liposome technologies may be infeasible because of the expense of production. Simple ligand-DNA complexes will not introduce genes into cells unless the receptors, against which the ligands are directed, internalize. Accordingly, currently available gene delivery systems impose severe limitations on the spectrum of diseases that can be treated by gene therapy.

Summary of the Invention

The invention features a method for expressing a nucleic acid of interest in a heterologous host cell. The method involves providing a virus whose genome comprises i) the nucleic acid of interest, and ii) a hybrid envelope gene. The hybrid gene encodes an envelope fragment joined to a targeting ligand, whereby the envelope fragment does not facilitate recognition or binding of its normal host cell but does facilitate efficient incorporation of the virus into a mature viral particle, and whereby the targeting ligand facilitates targeting and binding of the mature viral particle to the surface of the heterologous host cell. The method also involves administering the virus so as to permit viral infection of the cell.

By "efficient incorporation", is meant that the hybrid envelope protein is incorporated into a mature viral particle at least 25% as frequently as the corresponding wild-type envelope protein is incorporated into a mature viral particle.

In another aspect the invention features a virus, the genome of which encodes a hybrid envelope protein, wherein the hybrid protein comprises an envelope fragment joined in frame to a targeting ligand, whereby the envelope fragment does not facilitate recognition or binding of its normal host cell but which does facilitate efficient incorporation of the hybrid envelope protein into a mature viral particle and whereby the non-viral protein facilitates targeting and binding of the mature viral particle to the surface of a cell not normally infected by the virus.

In a third aspect the invention features a method for delivering a nucleic acid of interest to a heterologous host cell. The method involves providing a virus that comprises i) the nucleic acid of interest, and ii) a hybrid envelope gene, the hybrid gene encoding an envelope fragment joined to a targeting ligand, whereby the envelope fragment does not facilitate recognition or binding to its normal host cell but does facilitate efficient incorporation of the virus into a mature viral particle, and whereby the targeting ligand facilitates targeting and binding of the mature viral particle to the surface of the heterologous host cell. The method also involves administering the virus so as to permit viral infection of the cell.

In various preferred embodiments the virus is an enveloped virus, preferably a Herpesviridae, a Paramyxoviridae, or a Retroviridae, most preferably a Moloney murine leukemia virus, or the virus may also preferably be a Hepadnaviridae, a Poxviridae, or an Iridoviridae. Similarly the virus may be a Togaviridae, a Flaviviridae, a Coronaviridae, a Rhabodoviridae, a Filoviridae, an Orthomyxoviridae, a Bunyaviridae, or an Arenaviridae, or any other, yet unclassified, enveloped virus.

In other various preferred embodiments the nucleic acid of interest may include, without limitation, an antisense oncogene; a tumor suppressor gene, e.g., a gene encoding p53, or a gene encoding retinoblastoma protein Rb; a toxin gene, e.g., a diphtheria toxin gene; or a gene encoding a cytokine, e.g., a tumor necrosis factor, or an interferon. The nucleic acid of interest may be either DNA or RNA, e.g., antisense DNA, or antisense RNA, or a nucleic acid encoding an antisense RNA. The nucleic acid of interest may also be a gene invoking intracellular immunity, or a nucleic acid therapeutic for an inherited disease, e.g., an insulin gene, or a cystic fibrosis transmembrane regulator gene. A "gene that invokes intracellular immunity" is a gene that confers a dominant negative resistant phenotype to the cell it is in, thereby protecting the cell against an invading agent.

The heterologous host cell may be a cell that has acquired mutations that result in a disease state, preferably a cancer cell, e.g., a colon cancer cell. The heterologous host cell may be a cell infected with a second virus, e.g., a human immunodeficiency virus (HIV), a cell infected with an organism, or an infectious agent such as a bacterium or parasite. The infectious agent may be either unicellular or multicellular. The heterologous host cell may also be a cell affected by a hereditary disease, e.g., a pancreatic beta cell, or a lung cell.

The targeting ligand, in additional various preferred embodiments, may include a protein, preferably a hormone, or an immunoglobulin, more preferably an anti-tumor associated antigen-specific immunoglobulin, most preferably an anti-carcinoembryonic antigen-specific immunoglobulin, or an anti-HIVgp120 antigen-specific immunoglobulin. The targeting ligand may also be a

carbohydrate, or a lipid. The hybrid envelope fragment may consist of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, a signal sequence, and preferably a fusion domain. In some cases the fusion activity of the envelope fragment may be performed by a second protein. The second protein would therefore direct fusion of the virus with the membrane of the targeted cell.

The mode of administration may include, but is not limited to, 1) direct injection of the purified virus; or 2) implanting a container enclosing the virus into a patient. When the virus is administered inside a container, the virus is preferably inside a packaging cell. A "packaging cell" is a cell that supplies viral proteins necessary for production of viral vectors. By "container" is meant a virus permeable enclosure containing virus, or containing packaging cells with virus therein.

"Normal host cell" as used herein, is a cell type commonly infected by the naturally occurring virus. In contrast, the term "heterologous host cell" or a "targeted cell", as used herein, refers to a cell that is recognized as a function of the targeting ligand portion of the hybrid envelope protein, but is not recognized as a function of the envelope portion of the hybrid envelope protein. By "targeting ligand" is meant a molecule that has binding affinity for a molecule on the surface of a desired targeted cell. A "hybrid envelope protein", as used herein, is a protein that includes a portion of a viral envelope protein (or a biologically active analog thereof) covalently linked to a targeting ligand. For example, by a "hybrid immunoglobulin-*env* protein" is meant a portion of an immunoglobulin covalently linked to a portion of an envelope protein. A "hybrid envelope

gene" is a nucleic acid that provides genetic instructions for a hybrid envelope protein. By "hybrid anti-carcinoembryonic antigen-specific immunoglobulin" is meant a hybrid immunoglobulin-*env* protein that specifically binds to a carcinoembryonic antigen.

The term "fragment", as applied to an envelope protein fragment, includes some but not all of the envelope protein. A fragment will ordinarily be at least about about 20 amino acids, typically at least about 30 amino acids, usually at least about 40 contiguous amino acids, preferably at least about 50 amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Fragments of an envelope protein can be generated by methods known to those skilled in the art (e.g., those described herein).

A biologically active fragment of a viral envelope protein is one that possesses at least one of the following activities: a) it can bind to a cell membrane if given the appropriate targeting ligand; b) it can enable fusion with a cell membrane; or c) it can enable incorporation of proteins into a mature viral particle. These three biological activities can be performed by the same envelope protein fragment, or by two separate envelope protein fragments. As stated above, the envelope fragments of this invention do not facilitate recognition or binding of the virus' normal host cell. This is accomplished by either destroying the activity of the normal receptor binding region by mutation, or by physically deleting it. A new recombinant receptor-binding region is added in its place. The ability of a candidate fragment to exhibit a biological activity of a viral envelope protein can be assessed by methods known to those skilled in the art.

The envelope fragment may include the amino acid sequence of a naturally-ocurring viral envelope or may be

a biologically-active analog thereof. The biological activity of an envelope analog is assessed using the methods described herein for testing envelope fragments for activity.

Applicants have provided an efficient and reliable means for specifically delivering therapeutic genes or antisense nucleic acids to particular animal, plant or human cell types, or to cells of infectious agents. Their method facilitates treatments for mutagenically acquired, infectious, or inherited diseases, e.g., by either 1) antagonizing the effect of an existing cellular gene; 2) complementing the defect of an existing cellular gene; 3) destroying the target cells through the introduction of new genetic material; or 4) changing the phenotype of the target cells through the introduction of new genetic material. To specifically target cells for delivery, a hybrid envelope protein (e.g., an envelope-antibody or envelope-ligand hybrid) is utilized which directs specific interaction with a particular target host cell. The viruses itself, through its efficient internalization mechanisms, facilitates efficient uptake of the therapeutic gene. Such viral vectors are uniquely adapted to deliver genes, RNA, or drugs to cell surface proteins that do not normally internalize.

Another advantage of this invention is that it overcomes the problem of gene regulation encountered with other methods of gene therapy. Genetically-altered cells must not only synthesize the gene products at the right location, at the right time, and in the right amounts, but must also be regulated in the same manner as the indigenous tissue. That is, the transduced cells must also have all the proper signal transduction mechanisms to respond to extracellular signals. This may be a problem in gene therapy for diabetes, for example, where transplanting fibroblasts with insulin genes can be

ineffective or even harmful. As fibroblasts do not contain the same receptors and signal transduction machinery as pancreatic beta cells, the insulin genes may be expressed differently. Targeting genes to the right cells insures that the genes will be properly regulated.

In an additional aspect of the invention, a selection scheme is devised for creation of hybrid envelope protein-containing viruses. This strategy will be feasible for *env* proteins fused with immunoglobulins or with any ligand that recognizes specific receptors on cells.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings will first briefly be described.

Drawings

FIG. 1 is a representation of a scheme for constructing retroviral vector pLNCX*.

FIG. 2 is a representation of a scheme for constructing plasmid LNC*env*pA.

FIG. 3 is a representation of a scheme for constructing plasmid LNC*env*.

FIG. 4 is a representation of a scheme for constructing plasmid pUC Star-*Sig*.

FIG. 5 is a representation of a scheme for constructing plasmid LNC-*Sig*.

FIG. 6 is a representation of a scheme for constructing plasmid LNC-*anti*CEA.

FIG. 7 is a representation of plasmids used in the construction of targeted viruses.

FIG. 8 is a representation of a strategy for generating targeted retroviruses involving construction of hybrid immunoglobulin-*env* genes *in vitro*.

FIG. 9 is a representation of a strategy for generating targeted retroviruses involving generation of pooled virus constructions.

FIG. 10 is a representation of a strategy for generating targeted retroviruses involving selection and characterization of targeted virus.

FIG. 11 is a representation of a plasmid containing a targeted retroviral vector.

FIG. 12 is a representation of a scheme for constructing plasmid pUC Star-antiCEA.

FIG. 13 is a representation of an alternative scheme for constructing plasmid LNC-*anti*CEA.

What follows is a procedure for the delivery of genes to target cells using targeted viral vectors. To create and target a virus, the receptor recognition domain of the viral envelope protein is replaced with a ligand directed against a specific cell surface receptor. The hybrid envelope protein is incorporated into the viral envelope during the budding process, producing a hybrid virus *in vivo*. Upon infection of a host, the hybrid virus specifically recognizes its target cell and resultant fusion with that cell facilitates internalization (into the target cell) of viral genes, including the therapeutic gene(s) which are engineered into the viral genome. Such internalization can be extremely important; for example, immunotoxins, although efficient at delivering toxin molecules to target cells, are often clinically ineffective since the cell surface molecules to which they are targeted do not internalize, and internalization is required for entry of the toxin molecules into the cells (Waldmann, T.A., 1991, *Science* 252: 1657-1662). Targeted viruses circumvent this requirement for receptor internalization since the virus itself contains the necessary cell fusion machinery (Gilbert, J.M., et al., 1990, *J Virol* 64: 5106-5113;

Roizman, B. et al., 1990, in BN Fields, et al., eds. Virology, Raven Press, Ltd. New York).

General Requirements for Targeted Viruses

In general targeted viruses are constructed by replacing the receptor recognition domain of the viral envelope protein with a ligand directed against a specific cell surface receptor. The ligand can be, without limitation, an immunoglobulin (e.g., FAb, dAb, Fd, or Fc), a hormone, or any other synthetic or natural protein that can direct the binding of the targeted viruses to a cell surface molecule. The ligand is biologically incorporated into the viral envelope by genetic fusion with that portion of the normal viral envelope protein involved in viral assembly and budding. The envelope portion of the hybrid protein consists of an envelope fragment (or analog thereof) that is sufficient to direct efficient incorporation of the envelope hybrid protein into the viral envelope. Preferably, the envelope hybrid protein no longer directs an interaction between the virus and its normal host cell.

It has been demonstrated that changing the receptor specificity of the envelope protein of a virus changes the virus's tropism. For example, vesicular stomatitis virus (VSV) pseudotypes that have their virus envelope replaced with that of a retrovirus acquire the ability to infect retrovirus infectable cells (Schnitzer, T.J., et al., 1977, *J Gen Virol* 23:449-454; Zavada. J., et al., 1972, *J Gen Virol* 15:183-191), indicating that species specific protein-protein interactions between a virus core protein and an envelope protein are not critical for virus fusion and penetration at least in these cases. Moreover, previous experiments have also indicated that virus envelopes can tolerate changes in length or conformation introduced into the envelope protein, e.g., by a conjugated ligand. For example,

Gitman *et al.* have shown that Sendai virus envelopes reconstituted with viral envelope glycoproteins, chemically cross-linked to anti-erythrocyte antibodies acquire the ability to bind to erythrocytes that had been stripped of the normal virus receptor. Similarly, Sendai virus envelopes reconstituted with envelope proteins, chemically cross-linked to insulin molecules, were able to bind to receptor-stripped erythrocytes expressing the insulin receptor. In both cases, envelope binding but not fusion occurred with the receptor-stripped erythrocytes. Fusion between the conjugated envelopes and erythrocytes occurred, however, when the conjugated-envelopes were coreconstituted with the normal viral hemagglutinin/neuraminidase and fusion proteins (Gitman, A.G., et al., 1985, *Biochem* 24:2762-2768).

Preferably, the targeted virus contains 1) a viral envelope derived from a host cellular membrane; 2) a transmembrane hybrid envelope protein that directs the binding and penetration of the virus to specific target cells; 3) a transmembrane envelope protein that directs the fusion of the targeted virus with the cellular membrane of the targeted cell for viral penetration (e.g., the targeting protein itself or another envelope protein); 4) viral core proteins; 5) a foreign gene(s) of interest; and 6) all necessary viral and genetic components for penetration and expression of genes contained in the viral genome. The transmembrane hybrid envelope protein consists of 1) determinants that enable the hybrid protein to become processed and incorporated into viral envelopes; 2) determinants that enable fusion of the viral envelope with the targeted cellular membrane; these are essential for penetration of the targeted virus; 3) a ligand determinant that enables the targeted virus to recognize and bind to specific receptors on target cells. The viral genome may also

include bacterial selectable markers (e.g., ampicillin resistance) and/or a mammalian cell selectable marker (e.g., neomycin resistance).

The transmembrane hybrid protein is constructed genetically by splicing the cell surface receptor binding domain of a ligand gene to a portion of the viral envelope protein gene. The transmembrane hybrid protein must retain those portions of the envelope protein that direct the efficient post-translational processing, sorting and incorporation of the protein into the viral envelope.

The following domains must be considered in constructing the hybrid ligand-envelope protein:

1. The receptor binding domain

The receptor binding domain is that portion of the envelope protein that recognizes and binds to cell surface receptors. In hybrid envelope proteins, this portion of the envelope protein is replaced with ligand sequences. The receptor binding domain of retrovirus envelope proteins has been localized to the SU subunit (Coffin, J.M., 1990, in BN Fields, et al., eds. Virology, Raven Press, Ltd., New York). Since the SU protein of retroviruses is coded for 5′ to the transmembrane protein, replacement of the amino-terminal sequences of the envelope protein with ligand sequences poses no problem for the creation of a functional hybrid ligand-envelope protein.

2. Proteolytic cleavage site

The envelope protein of retroviruses is synthesized as a polyprotein which is later proteolytically cleaved to form SU and TM heterodimers. In construction of the hybrid ligand-envelope protein, the proteolytic cleavage site should be eliminated. The proteolytic cleavage site should be eliminated either by deletion or by site directed mutagenesis. Perez and

Hunter have demonstrated that elimination of the proteolytic cleavage site does not block transport or surface expression of Rous sarcoma virus envelope proteins (Perez, L.G., et al., 1987, *J Virol* 61:1609-1614).

3. Oligomerization domain

The envelope proteins of many animal viruses associate to form trimers (Fields, B.N., et al., 1991, Virology, 2nd ed., Raven Press, Ltd., New York). Trimerization of the envelope protein is thought to be essential for the proper transport and insertion of envelope proteins into the viral envelope (Singh, J. et al., 1990, *Embo J* 9:631-639; Kreis, T.E., et al., 1986, *Cell* 46:929-937). Therefore it is important that this domain be retained in the hybrid ligand-envelope protein. The trimerization domain likely resides in the transmembrane TM protein of retroviruses (Einfeld, D., et al., 1988, *Proc Natl Acad Sci USA* 85:8688-8692); hence, creation of a functional hybrid ligand-envelope retroviral protein lacking the SU subunit is possible. Some viral envelope proteins may oligomerize to form stoichiometric combinations other than trimers.

4. Fusion domain

The fusion domain is a hydrophobic stretch of amino acids that is involved in fusion of the virus envelope with the cell membrane (Wiley, D.C., et al., 1990, in Fields, B.N. et al., eds. Virology, 2nd ed., Raven Press, Ltd., New York). Viral fusion allows entry of the viral core proteins and genome into the cell. In influenza virus, the fusion domain, located in the amino terminus of the envelope HA2 protein, is sequestered in the hemagglutinin trimer until a low pH-induced conformational change allows presentation of the fusion domain to the cell membrane. Trimerization of the envelope proteins can prevent constitutive expression of

fusion activity by sequestering it within an internal hydrophobic pocket. A potential fusion domain has been located within the extracellular portion of the gp37 TM protein of Rous sarcoma virus (Hunter E. et al., 1983, *J Virol* 46:920-936). Similar hydrophobic fusion sequences have been noted in the p15E protein of Moloney murine leukemia virus (Mo-MuLV) (Chambers, P., et al.,1990, *J Gen Virol* 71:3075-3080).

In constructing a hybrid ligand-envelope protein, it may be necessary to eliminate the fusion domain to prevent the possibility of constitutive fusion activity, a state that may impair the infectivity of targeted viruses. Therefore two proteins may be incorporated into the viral envelope of targeting viruses. The first protein is the hybrid ligand-envelope protein which directs targeting of the virus but lacks fusion activity. The second protein is an envelope protein possessing fusion activity but lacking a receptor binding domain. This type of situation is observed for paramyxoviruses where one envelope protein is dedicated to targeting while another carries out fusion (Kingsbury, D.W., 1990, B.N. Fields, et al., eds. Virology, 2nd ed., Raven Press, Ltd., New York.). Where it is not necessary to prevent constitutive fusion activity, both activities may be included in one protein.

5. Transmembrane domain

The transmembrane domain is a stretch of approximately twenty or more amino acids that anchor the envelope protein to the viral envelope. It is located within the p15E protein of Moloney murine leukemia virus (Chambers, et al., *supra*). Retention of the transmembrane domain is thought to be essential since deletion of the transmembrane domain results in secretion of the synthesized envelope protein (Perez, L.G., et al., 1987, *J Virol* 61:2981-2988).

6. Virus budding domain

Amino acid sequences within the envelope protein may be involved with the exclusive incorporation of viral envelope proteins into viral envelopes and with virus budding. The virus budding domain directs the hybrid ligand-envelope protein into the viral envelope. These sequences are thought to reside within the portion of the envelope protein facing the inside of the virus and may involve specific protein-protein interactions between envelope proteins and viral core or matrix proteins. Although Perez *et al.* demonstrated that deletion of the carboxy-terminal sequences of the Rous sarcoma virus *env* protein resulted in normal budding of the mutant virus (Perez, L.G., et al., 1987, *J Virol* 61:2981-2988), evidence exists that, for other viruses, interactions between envelope proteins and viral core proteins may direct virus assembly and envelopment (BN Fields, et al., eds., 1990, Virology, Raven Press, Ltd., New York).

7. Sorting signals and other signals

Sorting signals are determinants that direct the envelope protein to the correct intracellular location during post-translational processing. These sequences insure that the envelope protein passes through the endoplasmic reticulum, Golgi apparatus, and other organelles until it eventually reaches the viral envelope. Other signals that may have to be retained in the hybrid ligand-envelope protein are glycosylation sequences and sequences involved in effective conformation of the envelope protein (e.g., disulfide bonds).

8. Signal sequence

The signal sequence is an amino-terminal hydrophobic stretch of amino acids that directs the envelope protein into the endoplasmic reticulum. The signal sequence, which is later proteolytically cleaved,

is essential for the hybrid ligand-envelope protein to become located in a membrane.

The diversity of signals and domains that must be considered in constructing targeted viruses requires that precise and correct splicing of ligand and envelope genes occur. The present invention describes a selection scheme for constructing targeted viruses whereby the ligand gene is spliced to an envelope gene fragment; this hybrid gene codes for those portions of the envelope protein which are required to direct efficient incorporation of the resultant hybrid envelope-ligand protein into the mature viral particle. According to the selection scheme, cell surface receptor binding domains of ligand genes are randomly ligated to progressive deletions of viral envelope genes. The correct combination of ligand and envelope sequences is determined by a selection scheme for the production of biologically active targeted virus. The selection scheme not only produces targeted virus but simplifies the construction of future targeted viruses.

A Specific Example of a Targeted Retrovirus

There now follows an example of a recombinant retrovirus which targets and infects particular host cells for the purpose of delivering to those cells a desired therapeutic gene. This example is provided for the purpose of illustrating, not limiting, the invention.

Moloney murine leukemia virus (Mo-MuLV) is a mouse ecotropic retrovirus. A recombinant Mo-MuLV based retroviral vector that is targeted to colon cancer cells is constructed. The targeted retroviral vector delivers the neomycin resistance gene to colon cancer cells. Targeting to human colon cancer cells is accomplished by incorporating into the viral envelope hybrid immunoglobulin-*env* proteins directed against carcinoembryonic antigen. Carcinoembryonic antigen (CEA)

is a tumor associated antigen expressed on the surface of human colon cancer cells but not on the surface of normal adult cells. The CEA glycoprotein, possessing multiple membrane spanning alpha helices, does not internalize in response to ligand (Benchimol, S. et al., 1989, *Cell* 57:327-334). A protein that is homologous to carcinoembryonic antigen has recently been shown to be the receptor for mouse hepatitis virus (Dveksler, G.S., et al, 1991, *J Virol* 65:6881-6891).

For the purpose of this illustration, a single variable region of the heavy chain of anti-CEA is fused to a portion of the *env* gene. Single variable heavy chain fragments (dAb) have been shown to be as effective in antigen binding as fragmented antibodies (FAb), containing both heavy and light chain fragments, and intact monoclonal antibodies (Ward, E.S., et al., *Nature* 341:544-546). The function of immunoglobulin-*env* proteins is not limited, however, to the use of dAb's and can be applied with FAb's, Fv's and mAb's.

Modification of the retroviral vector LNCX

LNCX is a Moloney murine leukemia virus based retroviral vector contained in the plasmid pLNCX (Miller, A.D., et al., 1989, *Biotechniques* 7:980-990). pLNCX contains a unique HindIII and ClaI cloning site for expression of inserted genes, a cytomegalovirus (CMV) promoter, a polyadenylation site (pA), retroviral long terminal repeats (LTR) for retroviral RNA transcription and reverse transcription, a bacterial neomycin resistance gene (Neo) which conveys resistance to both neomycin and G418, a bacterial origin of replication (Or), a bacterial ampicillin resistance gene (Amp), and a retroviral RNA packaging sequence (ψ+). LNCX is modified to contain a unique SalI site as shown in Figure 1. pLNCX is linearized with XbaI and subcloned into the XbaI site of the phagemid BluescriptII SK+ (Stratagene, La

Jolla, CA). Single stranded DNA is purified and the unique BstEII site of LNCX is converted into a SalI site by site directed mutagenesis with the oligonucleotide 5′-GCAGAAGGTCGACCCAACG-3′ (SEQ ID NO: 1). The BstEII site is located within the extended packaging signal (Ψ+) of Mo-MuLV RNA (Bender, M.A., et al., 1987, *J Virol* 61:1639-1646; Adam, M.A., et al., 1988, *J Virol* 62:3802-3806; Armentano, D. et al., 1987, *J Virol* 61:1647-1650). Conversion of the BstEII site to SalI does not affect packaging since this region has been determined to be dispensable for efficient packaging (Schwartzberg, P., et al., 1983, *J Virol* 46:538-546; Mann R. et al., 1985, *J Virol* 54:401-407; and Mann, R., et al., 1983, *Cell* 33:153-159). The BstEII site is converted into a SalI site because BstEII sites, but not SalI sites, frequently occur in heavy chain genes (Chaudhary, V.K., et al, 1990, *Proc Natl Acad Sci USA* 87:1066-1070). The SalI containing plasmid is recircularized with XbaI and DNA ligase to form the plasmid pLNCX*.

Cloning of the Mo-MuLV *env* protein in pLNCX*

The Mo-MuLV *env* gene is cloned into pLNCX* as shown in Figure 2. The Mo-MuLV *env* gene is excised from plasmid p8.2 (Shoemaker, C., et al., 1980, *Proc Natl Acad Sci USA* 77:3932-3936) as a 1.9kb ScaI-NheI fragment. The 1.9kb ScaI-NheI fragment contains the entire coding region for the p15E transmembrane protein and the majority of the coding region for the gp70 SU protein. The 5′-protruding ends are digested with S1-nuclease, and HindIII linkers (5-CCAAGCTTGG-3′; SEQ ID NO: 2) are added. The *env* gene is cloned as a HindIII fragment in the HindIII site of pLNCX* to form plasmid LNC*env*pA. The orientation of the HindIII *env* fragment is such that it can be transcribed and expressed from the cytomegalovirus (CMV) promoter.

Modification of LNC*envpA* to LNC*env*

LNC*envpA* is cloned as an XbaI fragment in phagemid pBluescript II SK+ for additional site directed mutagenesis (Figure 3). The *env* encoding HindIII fragment contains a polyadenylation signal that may interfere with the polyadenylation signal provided by the viral vector. The AAUAAA polyadenylation signal is therefore changed to AAGAAA by site directed mutagenesis with the oligonucleotide 5′-GTTTTCTTTTATC-3′ (SEQ ID NO: 3). The HindIII site located at the 3′ end of the *env* gene is eliminated by site directed mutagenesis with the oligonucleotide 5-CAAGCATGGCTTGCC-3′ (SEQ ID NO: 4). The *env* containing retroviral vector is recircularized by XbaI restriction and ligation to form plasmid LNC*env*.

Molecular cloning of anti-CEA immunoglobulin genes

cDNA encoding the mature variable region domain of anti-CEA heavy chain genes is cloned as an XhoI-SpeI fragment using the polymerase chain reaction (PCR) and RNA template. RNA is derived from the spleen of mice immunized against purified carcinoembryonic antigen. Alternatively, RNA can be derived from hybridoma cell lines that secrete monoclonal antibodies against CEA, e.g., 1116NS-3d (American Type Culture Collection CRL8019) or CEA 66-E3 (Wagener, C., et al., 1983, *J Immunol* 130:2308-2315).

The following PCR primers hybridize to cDNA encoding the aminoterminal end of mature heavy chain genes (Stratacyte, Inc.). The degenerate primers introduce an XhoI site which is underlined.

5′ AGGTGCAGCTGCTCGAGTCGGG 3′ (SEQ ID NO: 5)
5′ AGGTGCAACTGCTCGAGTCGGG 3′ (SEQ ID NO: 6)
5′ AGGTGCAGCTGCTCGAGTCTGG 3′ (SEQ ID NO: 7)
5′ AGGTGCAACTGCTCGAGTCTGG 3′ (SEQ ID NO: 8)
5′ AGGTCCAGCTGCTCGAGTCTGG 3′ (SEQ ID NO: 9)
XhoI

The following PCR primer hybridizes to immunoglobulin heavy chain mRNA within the region coding for the J-region and introduces SpeI and BstEII sites.

5′ CTATTAACTAGTGACGGTTACCGTGGTCCCTTGGCCCCA 3′ (SEQ ID NO: 10)

SpeI BstEII

The amplified anti-CEA variable heavy chain DNA is cloned as an XhoI-SpeI fragment in an ImmunoZAP H vector (Stratacyte, Inc.) (Mullinax, R.I. et al., 1990, *Proc Natl Acad Sci USA* 87:8095-8099). ImmunoZAP H is a modified lambdaZAP vector that has been modified to express in E.coli immunoglobulin variable heavy chain fragments behind a pelB signal sequence. The procedure could similarly be performed by expressing immunoglobulin variable light chain fragments in a packaging cell line.

Identification of high affinity anti-CEA clones

Clones expressing high affinity anti-CEA antibodies are identified by a filter binding assay. The anti-CEA phage library is screened by nitrocellulose plaque lifts with [^{125}I]bovine serum albumin conjugated to CEA, as previously described (Huse, W.D., et al., 1989, *Science* 246:1275-1281). High and intermediate affinity anti-CEA clones are chosen for further manipulation.

Construction of plasmid LNC-*immunoglobulin*

Two strategies are presented for creating plasmid LNC-*immunoglobulin* (in this example, LNC-*anti*CEA, which codes for an anti-CEA immunoglobulin gene). LNC-*immunoglobulin* vectors encode an immunoglobulin peptide

fused to an amino-terminal signal sequence. Some amino acids at the amino-terminal end of the mature immunoglobulin peptide have been modified by the PCR primers used to generate the immunoZAP library. Moreover, the design of the LNC-antiCEA plasmid results in the insertion of an extra amino acid at the amino terminal end. These amino acid changes do not affect antigen binding because 1) the amino acid changes are conservative; 2) the affected amino acids are normally variable at those sites; and 3) the affected amino acids occur within the framework region of immunoglobulins which has been shown not to participate in antigen binding or conformation of the antibody (Relchman et al. 1988 *Nature* 332:323-327). It is for these same reasons that cleavage of the signal sequence from mature peptide will not be affected.

Both strategies for creating LNC-*immunoglobulin* rely on the use of plasmid pUC Star-Sig, the construction of which is presented below.

An immunoglobulin signal sequence is cloned into a modified pUC119 vector to create pUC Star-*Sig* as follows (Figure 4). pUC119 is a phagemid containing a polylinker cloning site. The multiple cloning sites of pUC119 are replaced with new restriction sites by insertion of the following polylinker into the HindIII and XbaI sites of pUC119.

HindIII PstI XhoI BclI SpeI NotI ClaI Xbal
5′-AGCTTCTGCAGGCTCGAGTGATCAACTAGTGCGGCCGCATCGATT-3′ (SEQ ID NO: 11)
3′-AGACGTCCGAGCTCACTAGTTGATCACGCCGGCGTAGCTAAGATC-5′(SEQ ID NO:12)

The modified pUC119 is called pUC Star-1 (Figure 4). The restriction sites may be further separated by small

linkers, if adjacent restriction sites interfere with one another during digestion.

The signal sequence from an anti-NP immunoglobulin heavy chain gene is isolated from plasmid pcDFL.1 (Ucker, D.S., et al., 1985, *J Immnol* 135:4204-4214) as a ~330bp PstI fragment. The 330bp PstI fragment is subcloned into pUC Star-1 to yield plasmid pUC Star-*Sig* (Figure 4). The PstI fragment is oriented so that the signal sequence can be expressed.

A. Construction of LNC-*immunoglobulin* through plasmid LNC-*Sig*, an immunoglobulin expression vector.

LNCX* is converted into a eukaryotic immunoglobulin expression vector (Figures 4 and 5). An immunoglobulin heavy chain signal sequence and XhoI-SpeI cloning sites are inserted behind the CMV promoter of plasmid LNCX* to allow expression of the PCR amplified immunoglobulin genes. Conversion of LNCX* is as follows.

The immunoglobulin heavy chain signal sequence is recovered from pUC Star-*Sig* as a HindIII-ClaI restriction fragment and cloned into the HindIII-ClaI sites of LNCX*. The resulting plasmid, LNC-*Sig* contains a retroviral vector with the immunoglobulin heavy chain signal sequence under control of the CMV promoter (Figure 5).

An anti-CEA gene from the immunoZAP library is then subcloned into LNC-*Sig* to form plasmid LNC*anti*-CEA. This generates an anti-CEA variable heavy chain gene containing a signal sequence (Figure 6). The anti-CEA gene is first excised from immunoZAP phage DNA as a Bluescript SK- phagemid (see lambdaZAP protocols, Stratagene, Inc. La Jolla, CA). The anti-CEA gene is purified as an XhoI-SpeI fragment and ligated to XhoI-SpeI restricted LNC-*Sig*. LNC-*Sig* contains three SpeI sites. Therefore, to generate plasmid LNCanti-CEA, the ligation mix is transformed into neomycin-sensitive, ampicillin-sensitive E. coli and neomycin-resistant,

ampicillin-resistant transformants are selected for. Plasmid LNC*anti*-CEA is screened from neomycin-resistant, ampicillin-resistant transformants by using the SpeI-XhoI anti-CEA restriction fragment from Bluescript SK-anti-CEA as a probe. The SpeI site is used because of dependence upon the available sites in the ImmunoZap expression vector. To simplify construction of LNC*anti*-CEA, a unique NotI site can be introduced into the ImmunoZap H expression vector so that NotI sites can be used instead of SpeI sites.

B. Construction of LNC-*immunoglobulin* through plasmid pUC Star-*Sig*

An anti-CEA gene from the immunoZAP library is subcloned into plasmid pUC Star-*Sig* to form plasmid pUC Star-anti-CEA. This generates an anti-CEA variable heavy chain gene containing a signal sequence (Figure 12). The anti-CEA gene is excised from immunoZAP phage DNA as a Bluescript SK-phagemid (see lambdaZAP protocols, Stratagene, Inc., La Jolla, CA). Bluescript SK-anti-CEA double stranded DNA is prepared and restricted with XhoI and SpeI. The anti-CEA containing XhoI-SpeI fragment is purified by electroelution and ligated to Xho-SpeI restricted pUC Star-Sig to create plasmid pUC Star-antiCEA (Figure 12).

The antiCEA gene is transferred from pUC Star-anti CEA to LNCX* as a HindIII-ClaI fragment to create plasmid LNC-antiCEA (figure 13). The antiCEA-containing HindIII-ClaI fragment is purified from pUC Star-antiCEA by electroelution. Phosphatase treated, HindIII-ClaI restricted LNCX* is ligated with the purified HindIII-ClaI antiCEA fragment to generate LNC-antiCEA (figure 13).

Strategy for generating targeted viruses

The starting materials for generation of targeted viruses are the LNC*env* and LNC-immunoglobulin (in this

example, LNC-*anti*CEA) plasmids shown in Figure 7. Figures 8-11 diagram the general principle for the primary generation of targeted viruses. Hybrid immunoglobulin-*env* proteins are generated that target viruses to cells expressing carcinoembryonic antigen. Since the location of important determinants for envelope protein sorting (S), trimerization (T), and fusion (F) is not known with certainty, the immunoglobulin gene is ligated to progressive deletions of the *env* gene and functional immunoglobulin-*env* hybrids are selected for.

Useful Envelope Fragments or Analogs

The envelope portion of the fusion protein may consist of any portion of the envelope protein (or any analog thereof) which is sufficient to direct efficient incorporation of the envelope fusion protein into the viral coat (upon budding of the recombinant virus from a producer cell line). Such fragments or analogs may be determined using the following general selection scheme which generally involves ligation of cell surface receptor binding domains of ligand genes to progessive deletions of viral envelope genes. The correct combination of ligand and envelope sequences is determined by a selection scheme for the production of biologically active targeted virus. The selection scheme not only produces targeted virus but simplifies the construction of future targeted viruses.

Construction of hybrid immunoglobulin-*env* genes *in vitro*

Plasmid LNC*env* contains the coding region for the Mo-MuLV *env* polyprotein (Figure 8). LNC*env* is first linearized by HindIII restriction. A range of deletions extending into the *env* gene is created by collecting aliquots of Exonuclease III treated DNA over time and removing 5′-processive ends with S1-nuclease (Guo, I.H., et al., 1983, *Methods Enzymol* 100:60; and Sambrook, J., et al., 1989, Molecular Cloning, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor). NotI linkers (5′ AGCGGCCGCT 3′ SEQ ID NO: 13) are ligated onto the blunt end termini and restricted with NotI. This results in a NotI restriction overhang at the 5′-border of every deletion within the *env* gene. The NotI overhangs at the other end of the molecules are removed by SalI restriction of the reaction mixture. The reaction mixture is then treated with phosphatase to prevent circularization of the reaction products.

The reaction mixture is ligated to a SalI-NotI restriction fragment from LNC-*anti*CEA that contains the anti-CEA variable heavy chain gene. This creates a pool of functional retroviral vectors encoding an anti-CEA peptide fused to a series of *env* deletions.

Generation of pooled virus constructions

The total reaction mixture from above is transformed into ampicillin-sensitive E. coli and ampicillin resistance is selected for (Figure 9). Recombinants containing functional retroviral vectors are selected for since only they contain the ampicillin resistance gene. Plasmid DNA is prepared from transformants grown in liquid culture to create a pool of retroviral vectors containing different immunoglobulin-*env* fusion genes.

The DNA is transfected into the crip2 retroviral packaging cell line (Danos, O., et al., 1988, *Proc Natl Acad Sci USA* 85: 6460-6464). Alternatively, DNA is transfected into a packaging cell line that does not encode wild-type env protein. The transfected packaging cell line synthesizes each of the different hybrid immunoglobulin-*env* proteins as well as the wild type *env* protein (encoded by an *env* gene contained in the cell line). The transfected packaging cell line secretes a pool of enveloped retroviruses containing the different retroviral genomes encoding hybrid immunoglobulin-*env*

genes. If the hybrid immunoglobulin-*env* protein retained all of the necessary determinants for efficient incorporation into viral envelopes then the hybrid-*env* protein can be incorporated into viral envelopes. Wild type *env* proteins encoded for by the packaging cell line are also incorporated into the viral envelopes. This creates a virus containing both wild type and hybrid *env* proteins in the viral envelope. This system therefore selects for immunoglobulin-*env* hybrids that can incorporate their gene products into the viral envelope.

Virus pools are harvested from media filtered at 0.45μ to remove contaminating G418-resistant packaging cells.

Selection and characterization of targeted virus

G418-sensitive target cells are exposed to virus pools by standard procedures, and G418-resistant cells are selected for. The target cells can be any non-mouse cell line (uninfectable by wild type Mo-MuLV) that expresses carcinoembryonic antigen. Examples include ATCC COLO 205, a human cell line isolated from the ascites of a patient with carcinoma of the colon (A.T.C.C.#CCL 222); LR-73 CEA, a chinese hamster ovary cell line transfected with a mouse carcinoembryonic antigen gene (Benchimol, S. et al., *supra*); and HCT48, a human colon adenocarcinoma cell line (Shi, Z.R., et al., 1883, *Cancer Res* 43:4045-4049).

G418-resistant cells can only have arisen from transduction of the neomycin resistance gene by targeted virus. This system therefore selects for recombinant viruses that have hybrid immunoglobulin-*env* proteins that have retained all the necessary determinants for viral targeting and fusion.

Rescue of integrated immunoglobulin-*env* gene

Infection by targeted virus results in integration of the hybrid envelope gene that created the targeting

protein. The integrated hybrid immunoglobulin-*env* gene is rescued from the host DNA by polymerase chain reaction (PCR) with the following primers:

PCR 5′ Rescue primer:

5′-CCAGCCTCCGCGGCCCCAAGCTTCTGCA-3′ (SEQ ID NO: 14)

HindIII

PCR 3′ Rescue primer:

5′-GGTTCTCTAGAAACTGCTGAGGGC-3′ (SEQ ID NO: 15)

XbaI

PCR amplification with these primers generates the immnoglobulin-*env* gene bordered by HindIII and XbaI sites. The amplified DNA is restricted with HindIII and XbaI to create sticky ends and the DNA is ligated into HindIII-XbaI cut LNCX*. When transfected into crip2 packaging cells, this generates a retroviral vector targeted to cells expressing cell surface carcinoembryonic antigen (e.g., colon cancer cells).

The retroviral vector produced in the above selection scheme is targeted to both CEA-expressing human cells (directed by the hybrid envelope protein) and normal mouse cells (directed by the wild type envelope protein) when produced in crip2 packaging cells. To create viruses that infect target cells only, the retroviral vector will first be tested to determine if incorporation of the hybrid envelope protein alone is sufficient to direct virus fusion. This is accomplished by transfecting DNA into a modified packaging cell line that does not encode wild type *env*.

If fusion functions are found to have been supplied from the wild type envelope protein, targeted viruses will be created as follows. A packaging cell line will be created that encodes an *env* gene containing mutations in the receptor binding domain. When

transfected with the targeted viral vector DNA, targeted viruses expressing both hybrid ligand-*env* proteins and *env* proteins with mutated binding sites will be produced. The viruses will exclusively infect target cells.

The targeted viral vector is a universal vector

The viral vector that is constructed by the above procedure is a universal targeted vector (Figure 11). Targeting to other cells is accomplished by replacing the XhoI-SpeI anti-CEA fragment with any XhoI-SpeI fragment encoding an in-frame immunoglobulin or ligand directed against specific cell surface proteins. For example, an XhoI-SpeI immunoglobulin-containing fragment from an immunoZAP library can be fused in frame behind a signal sequence and subcloned into LNCX* through the pUC Star-Sig plasmid, as outlined above. Substituting a SalI-NotI fragment from another LNC-immunoglobulin plasmid into the universal vector would create another targeted virus vector.

Other Viral Vectors

Any enveloped virus may be used as a vector for the targeted delivery of a therapeutic gene. Particular examples include both DNA and RNA viruses, such as Herpesviridae, e.g., herpes simplex type 1 or 2, Paramyxoviridae, Retroviridae, Hepadnaviridae, Poxviridae, Iridoviridae, Togaviridae, Flaviviridae, Coronaviridae, Rhabodoviridae, Filoviridae, Orthomyxoviridae, Bunyaviridae, or Arenaviridae, or any other, yet unclassified, enveloped virus.

An extensive selection of these viruses is available, e.g., from the American Type Culture Collection.

Targeting Ligands

Any molecule that is capable of directing specific interaction with a target host cell (e.g., by specific recognition of and binding to a host cell surface

protein) may be used as the targeting ligand portion of the envelope fusion protein. Preferably, such a protein is derived from one member of a ligand:receptor pair. The targeting ligands are not limited to proteins. Carbohydrate and lipid moieties can be attached to the envelope protein via protein fragments containing consensus sequences for glycosylation and lipidation.

Immunoglobulin genes can be used as ligands, as shown in the example above. Genes for high affinity immunoglobulins are screened from a lambda or bacterial expression library by a filter binding assay with [^{125}I] bovive serum albumin conjugated to antigen, as previously described (Huse, et al. *Supra*).

Cell surface molecules such as integrins, adhesion molecules or homing receptors can be used as cell-specific ligands since they are involved in cell-cell interactions via receptors on other cells. Genes encoding these molecules can be identified by the panning method of Seed and Aruffo (Seed. B., et al., 1987, *Proc Natl Acad Sci USA* 84:3365-3369).

Hormones that bind to specific receptors can be used as targeting ligands as well as viral proteins, such as HIV envelope protein gp120, and modifications of naturally occuring ligands.

Therapeutic Genes

Therapeutic genes useful in the invention include the following. 1) Genes that are therapeutic to cancer cells may include a) antisense oncogenes; b) tumor suppressor genes, such as p53 or the retinoblastoma gene product *Rb*, c) destructive toxin genes such as a diphtheria toxin gene; d) cytokines such as tumor necrosis factor or interferons; or e) any other therapeutic gene. 2) Therapeutic genes targeted to cells that are infected with HIV. Specific examples include antisense DNA complementary to essential genes

for HIV, e.g., polymerase; destructive toxin genes; e.g., diphtheria toxin; and genes that will invoke intracellular immunity, e.g., HIV enhancer sequences that titrate and remove HIV regulatory proteins (Baltimore, D., 1988, *Nature* 335:395-396). 3) Genes to correct inherited deficiencies. Examples include, but are not limited to, insulin genes delivered specifically to pancreatic beta cells, or the cystic fibrosis transmembrane regulator (CFTR) gene delivered to the appropriate lung cells of cystic fibrosis patients. The expression of targeted genes can be further accomplished through the use of tissue specific enhancers that regulate the transgene.

Therapy

For any gene therapy described herein, the appropriate recombinant virus, as described above, is administered to a patient in a pharmaceutically-acceptable buffer (e.g., physiological saline). The therapeutic preparation is administered in accordance with the condition to be treated. For example, to treat an HIV-infected individual, the virus is administered by direct injection, e.g., by intravenous, intramuscular, or intraperitoneal injection, at a dosage that provides suitable targeting and lysis of HIV-infected host cells. Alternatively, it may be necessary to administer the targeted virus surgically to the appropriate target tissue, or via a catheter, or a videoscope. It may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or spray. Again, an appropriate dosage is an amount of therapeutic virus which effects a reduction in the disease.

Targeted virus can also be administered by implanting viral packaging cells into a patient. The cells can be enclosed in a semi-permeable container, e.g., permeable to a virus but not permeable to a

packaging cell. The implanted container may be removable. Alternatively, the container may be hooked up to a patient intravenously, so that virus enters the patient through a needle or through a catheter. In this way the patient receives a continuous dose of viral gene therapy.

Other Embodiments

Other embodiments are within the following claims.

For example, replication competent viruses may be used in certain cases. In other cases, where replication-deficient viruses are necessary, it may be efficacious to administer modified packaging cells, rather than the targeted virus, to patients. By this method a non-proliferating dose of recombinant virus is delivered to a local area, and then the virus locates the specific target cell. For example, tumor infiltrating lymphocytes (TIL), which surround cancer cells, can be modified to secrete locally high concentrations of cancer cell-targeted virus. Treatment may be repeated as necessary. Immune response against targeted viruses can be overcome with immunosuppressive drugs.

In addition to colon cancer cells, the virus of the invention may be used to target other cancer cells, e.g., ovarian, breast, or lung cancer cells, or cells affected with hereditary diseases such as muscular dystrophy, Huntington's disease, or cells with a defect in adenosine deaminase. Herpesviridae viruses may include Herpes simplex type 1 or type 2, Epstein-Barr virus, or Cytomegalovirus. Sendai virus and Vaccinia virus may also be adapted to this method.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: GENE THERAPY USING TARGETED VIRAL VECTORS

(iii) NUMBER OF SEQUENCES: 15

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(A) MEDIUM TYPE:	3.5" Diskette, 1.44 Mb
(B) COMPUTER:	IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM:	IBM P.C. DOS (Version 5.00)
(D) SOFTWARE:	WordPerfect (Version 5.1)

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	19
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCAGAAGGTC GACCCAACG 19

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	10
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCAAGCTTGG 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	13
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTTTTCTTTT ATC 13

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	15
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CAAGCATGGC TTGCC 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGGTGCAGCT GCTCGAGTCG GG 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGTGCAACT GCTCGAGTCT GG 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGGTGCAGCT GCTCGAGTCT GG 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AGGTGCAACT GCTCGAGTCT GG 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	22
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGGTCCAGCT GCTCGAGTCT GG 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	39
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTATTAACTA GTGACGGTTA CCGTGGTCCC TTGGCCCCA 39

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	45
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGCTTCTGCA GGCTCGAGTG ATCAACTAGT GCGGCCGCAT CGATT 45

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	45
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGACGTCCGA GCTCACTAGT TGATCACGCC GGCGTAGCTA AGATC 45

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	10
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGCGGCCGCT 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCAGCCTCCG CGGCCCCAAG CTTCTGCA 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	24
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGTTCTCTA GAAACTGCTG AGGGC 24

What is claimed is:

Claims

1. A method for expressing a nucleic acid of interest in a heterologous host cell, said method comprising

(a) providing a virus whose genome comprises (i) said nucleic acid of interest and (ii) a hybrid envelope gene, said hybrid gene encoding an envelope fragment joined to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding of its normal host cell but which does facilitate efficient incorporation of said virus into a mature viral particle and whereby said targeting ligand facilitates targeting and binding of said mature viral particle to the surface of said heterologous host cell, and

(b) administering said virus so as to permit viral infection of said heterologous host cell.

2. The method of claim 1, wherein said virus is an envelope virus.

3. The method of claim 2, wherein said envelope virus is a Herpesviridae.

4. The method of claim 2, wherein said envelope virus is a Retroviridae.

5. The method of claim 4, wherein said Retroviridae is a Moloney murine leukemia virus.

6. The method of claim 1, wherein said nucleic acid of interest is DNA.

7. The method of claim 1, wherein said nucleic acid of interest is RNA.

8. The method of claim 1, wherein said heterologous host cell is infectious.

9. The method of claim 1, wherein a portion of said hybrid envelope fragment consists of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

10. The method of claim 9, wherein said envelope fragment further consists of a fusion domain.

11. The method of claim 1, wherein the fusion activity of said envelope fragment is performed by a second protein.

12. The method of claim 1, wherein said administration is by implanting a container enclosing said virus into a patient.

13. The method of claim 12, wherein said virus is inside a packaging cell.

14. A virus, the genome of which encodes a hybrid envelope protein, said hybrid protein comprising an envelope fragment joined in frame to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding of its normal host cell but which does facilitate efficient incorporation of said hybrid envelope protein into a mature viral particle and whereby said non-viral protein facilitates targeting and binding of said mature viral particle to the surface of a cell not normally infected by said virus.

15. The virus of claim 14, wherein said virus is an envelope virus.

16. The virus of claim 15, wherein said envelope virus is a Herpesviridae.

17. The virus of claim 15, wherein said envelope virus is a Retroviridae.

18. The virus of claim 17, wherein said Retroviridae is a Moloney murine leukemia virus.

19. The virus of claim 14, wherein said nucleic acid of interest is DNA.

20. The virus of claim 14, wherein said nucleic acid of interest is RNA.

21. The virus of claim 14, wherein said heterologous host cell is infectious.

22. The virus of claim 14, wherein a portion of said hybrid envelope protein consists of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

23. The virus of claim 22, wherein said envelope fragment further consists of a fusion domain.

24. The virus of claim 14, wherein the fusion activity of said envelope fragment is performed by a second protein.

25. The virus of claim 14, wherein said administration is by implanting a container enclosing said virus into a patient.

26. The virus of claim 25, wherein said virus is inside a packaging cell.

27. A method for delivering a nucleic acid of interest to a heterologous host cell, said method comprising

a) providing a virus whose genome comprises (i) said nucleic acid of interest and (ii) a hybrid envelope gene, said hybrid gene encoding an envelope fragment joined to a targeting ligand, whereby said envelope fragment does not facilitate recognition or binding to its normal host cell but does facilitate efficient incorporation of said virus into a mature viral particle and whereby said targeting ligand facilitates targeting and binding of said mature viral particle to the surface of said heterologous host cell, and

b) administering said virus so as to permit viral infection of said cell.

28. The method of claim 27, wherein said virus is an envelope virus.

29. The method of claim 28, wherein said envelope virus is a Herpesviridae.

30. The method of claim 28, wherein said envelope virus is a Retroviridae.

31. The method of claim 30, wherein said Retroviridae is a Moloney murine leukemia virus.

32. The method of claim 27, wherein said nucleic acid of interest is DNA.

33. The method of claim 27, wherein said nucleic acid of interest is an RNA.

34. The method of claim 27, wherein said heterologous host cell is infectious.

35. The method of claim 27, wherein a portion of said hybrid envelope fragment consists of a receptor binding domain, an oligomerization domain, a transmembrane domain, a virus budding domain, sorting signals, and a signal sequence.

36. The method of claim 35, wherein said envelope fragment further consists of a fusion domain.

37. The method of claim 27, wherein said the fusion activity of said envelope fragment is performed by a second protein.

38. The method of claim 27, wherein said administration is by implanting a container enclosing said virus into a patient.

39. The method of claim 38, wherein said virus is inside a packaging cell.

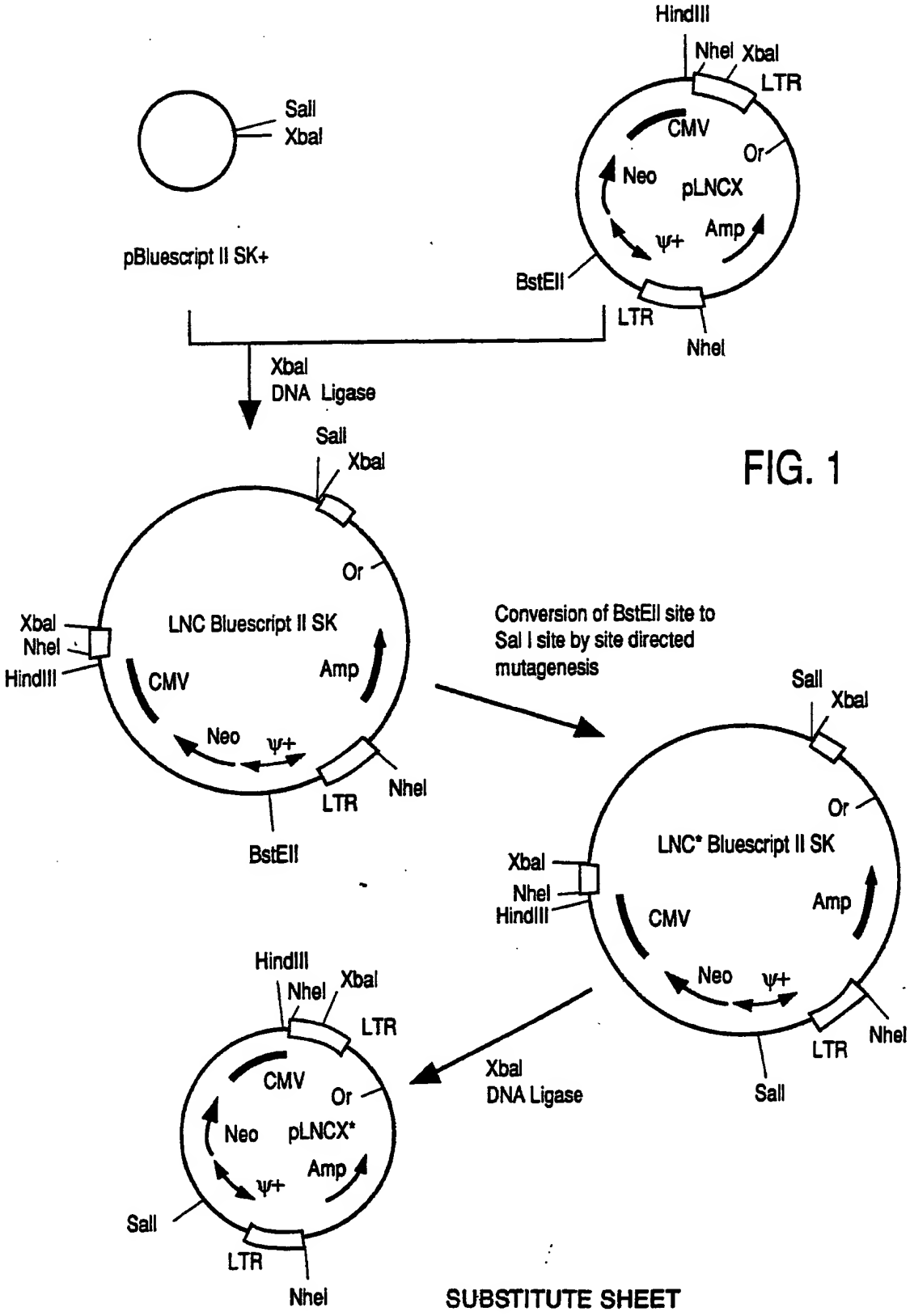

HindIII
NheI XbaI
LTR
CMV
Or
Neo
pLNCX
Amp
ψ+
BstEII
LTR
NheI
SalI
XbaI
pBluescript II SK+
XbaI
DNA Ligase
SalI
XbaI
Or
XbaI
NheI
HindIII
LNC Bluescript II SK
CMV
Amp
Neo
ψ+
LTR
NheI
BstEII
Conversion of BstEII site to
Sal I site by site directed
mutagenesis
SalI
XbaI
Or
XbaI
NheI
HindIII
LNC* Bluescript II SK
CMV
Amp
Neo
ψ+
LTR
NheI
SalI
HindIII
NheI
XbaI
LTR
CMV
Or
Neo
pLNCX*
Amp
ψ+
SalI
LTR
NheI
XbaI
DNA Ligase

FIG. 1

FIG. 2

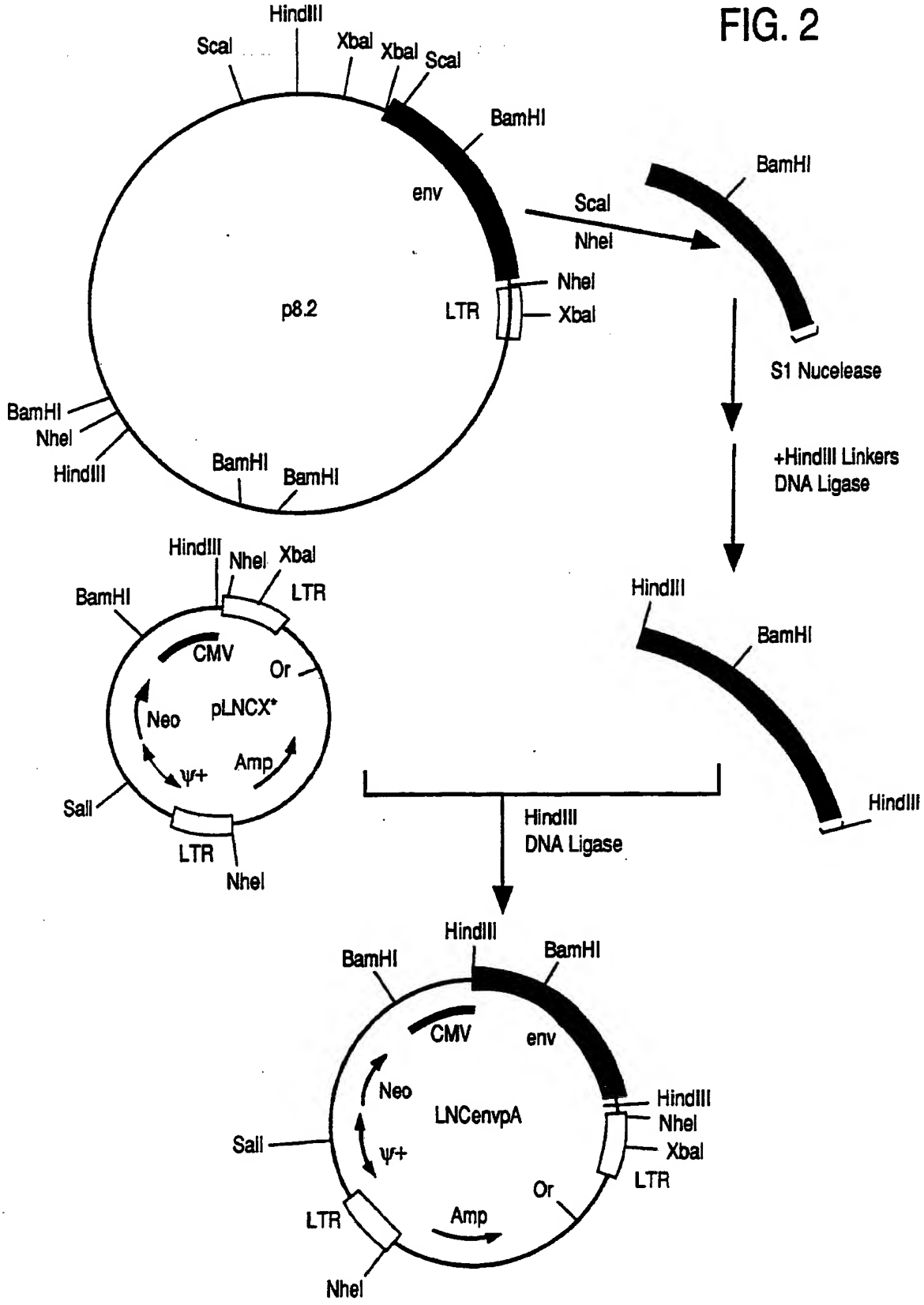
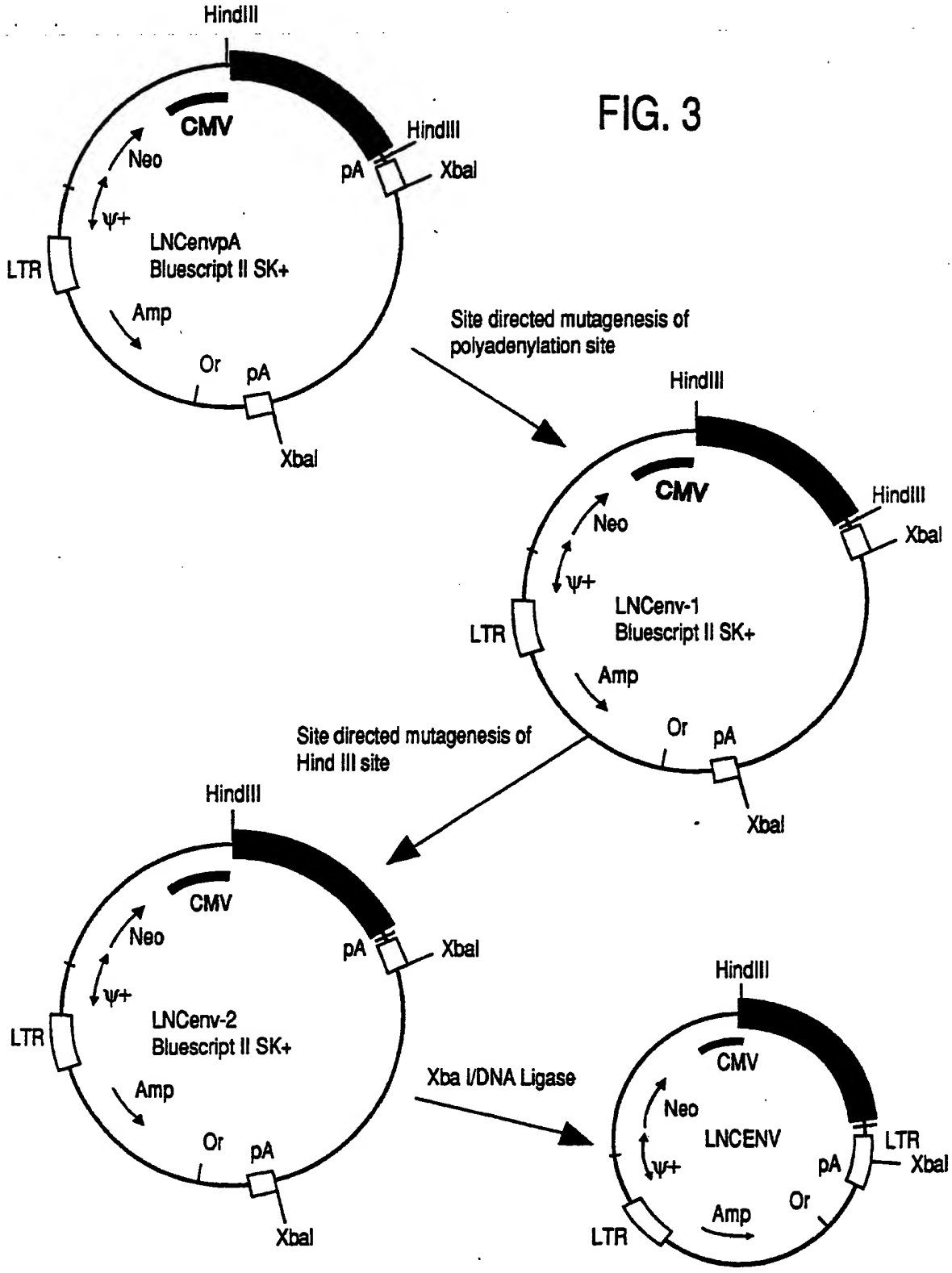

HindIII
ScaI
XbaI
XbaI
ScaI
BamHI
env
p8.2
LTR
NheI
XbaI
BamHI
NheI
HindIII
BamHI
BamHI
ScaI
NheI
BamHI
S1 Nucelease
+HindIII Linkers
DNA Ligase
HindIII
BamHI
HindIII
HindIII
NheI
XbaI
BamHI
LTR
CMV
Or
Neo
pLNCX*
Ψ+
Amp
SalI
LTR
NheI
HindIII
DNA Ligase
HindIII
BamHI
BamHI
CMV
env
Neo
LNCenvpA
Ψ+
SalI
HindIII
NheI
XbaI
LTR
Or
LTR
Amp
NheI

FIG. 3


HindIII
CMV
Neo
ψ+
LTR
LNCenvpA
Bluescript II SK+
Amp
Or
pA
HindIII
pA
XbaI
XbaI
Site directed mutagenesis of
polyadenylation site
HindIII
CMV
Neo
ψ+
LTR
LNCenv-1
Bluescript II SK+
Amp
Or
pA
HindIII
XbaI
XbaI
Site directed mutagenesis of
Hind III site
HindIII
CMV
Neo
ψ+
LTR
LNCenv-2
Bluescript II SK+
Amp
Or
pA
pA
XbaI
XbaI
Xba I/DNA Ligase
HindIII
CMV
Neo
ψ+
LNCENV
LTR
Amp
Or
pA
LTR
XbaI

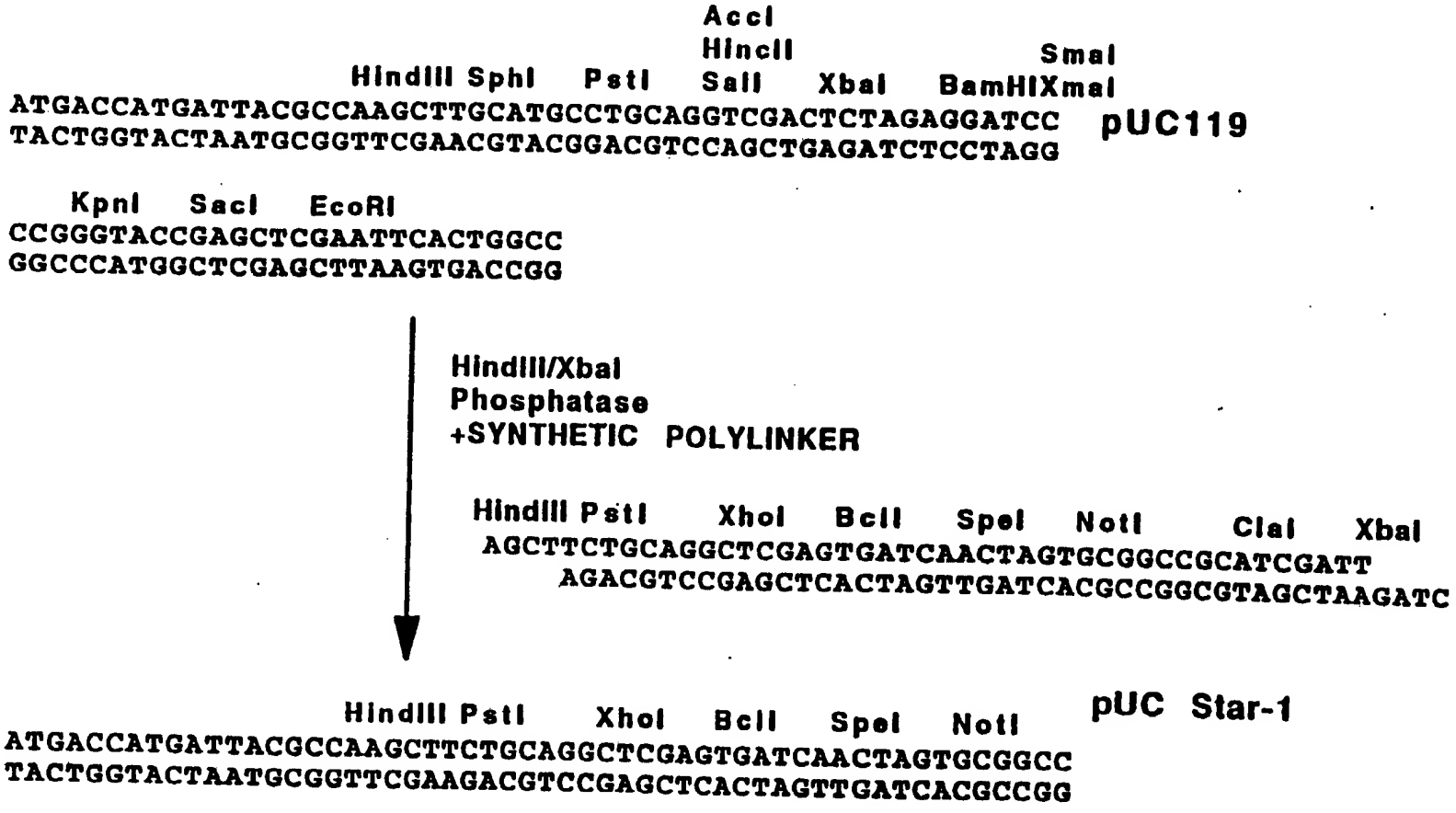
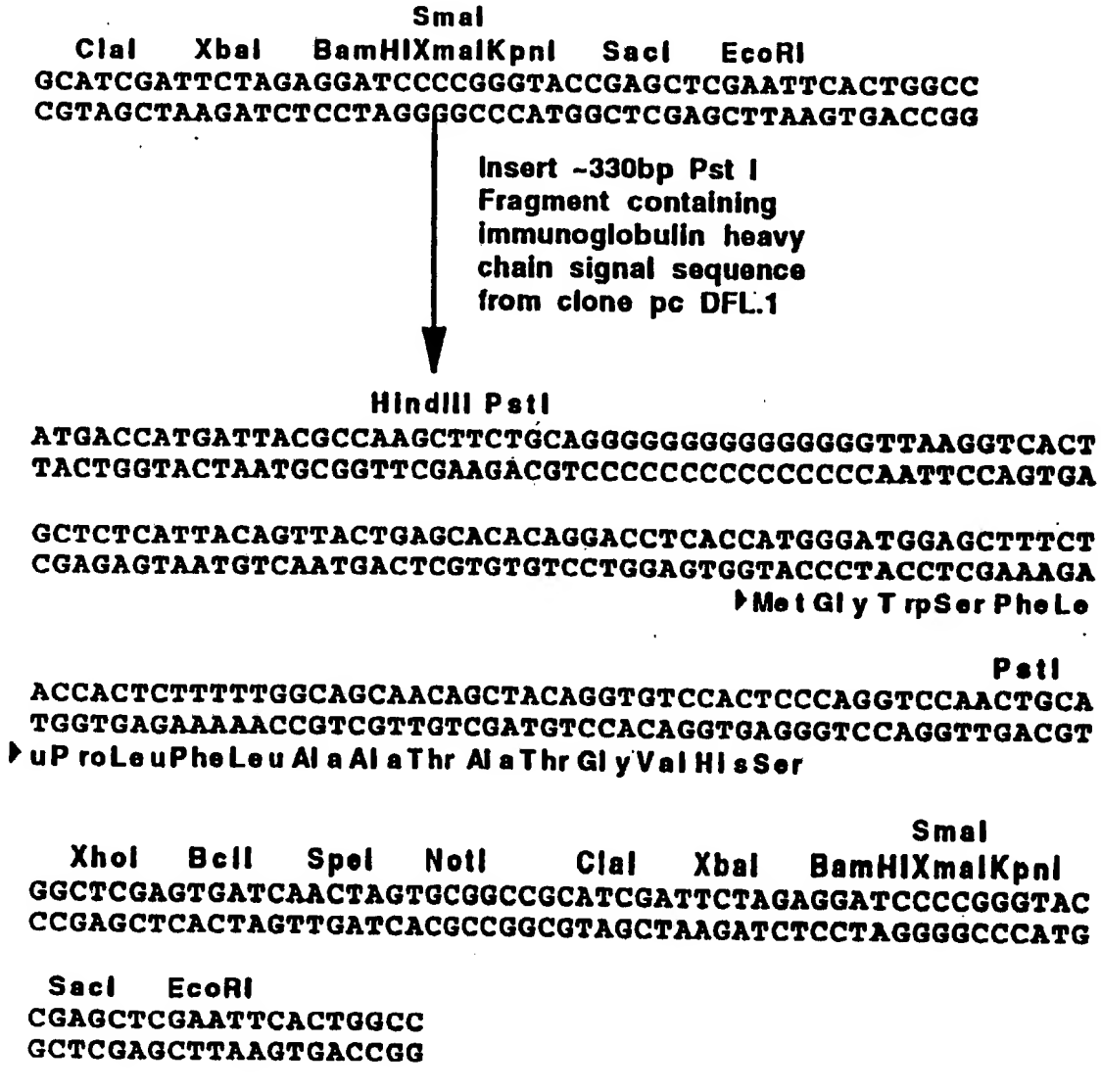

AccI
HincII
SmaI
HindIII SphI PstI SalI XbaI BamHI XmaI
ATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCC
TACTGGTACTAATGCGGTTCGAACGTACGGACGTCCAGCTGAGATCTCCTAGG
pUC119
KpnI SacI EcoRI
CCGGGTACCGAGCTCGAATTCACTGGCC
GGCCCATGGCTCGAGCTTAAGTGACCGG
HindIII/XbaI
Phosphatase
+SYNTHETIC POLYLINKER
HindIII PstI XhoI BclI SpeI NotI ClaI XbaI
AGCTTCTGCAGGCTCGAGTGATCAACTAGTGCGGCCGCATCGATT
AGACGTCCGAGCTCACTAGTTGATCACGCCGGCGTAGCTAAGATC
HindIII PstI XhoI BclI SpeI NotI
pUC Star-1
ATGACCATGATTACGCCAAGCTTCTGCAGGCTCGAGTGATCAACTAGTGCGGCC
TACTGGTACTAATGCGGTTCGAAGACGTCCGAGCTCACTAGTTGATCACGCCGG

FIG. 4A



pUC Star-*Sig*

FIG. 4B

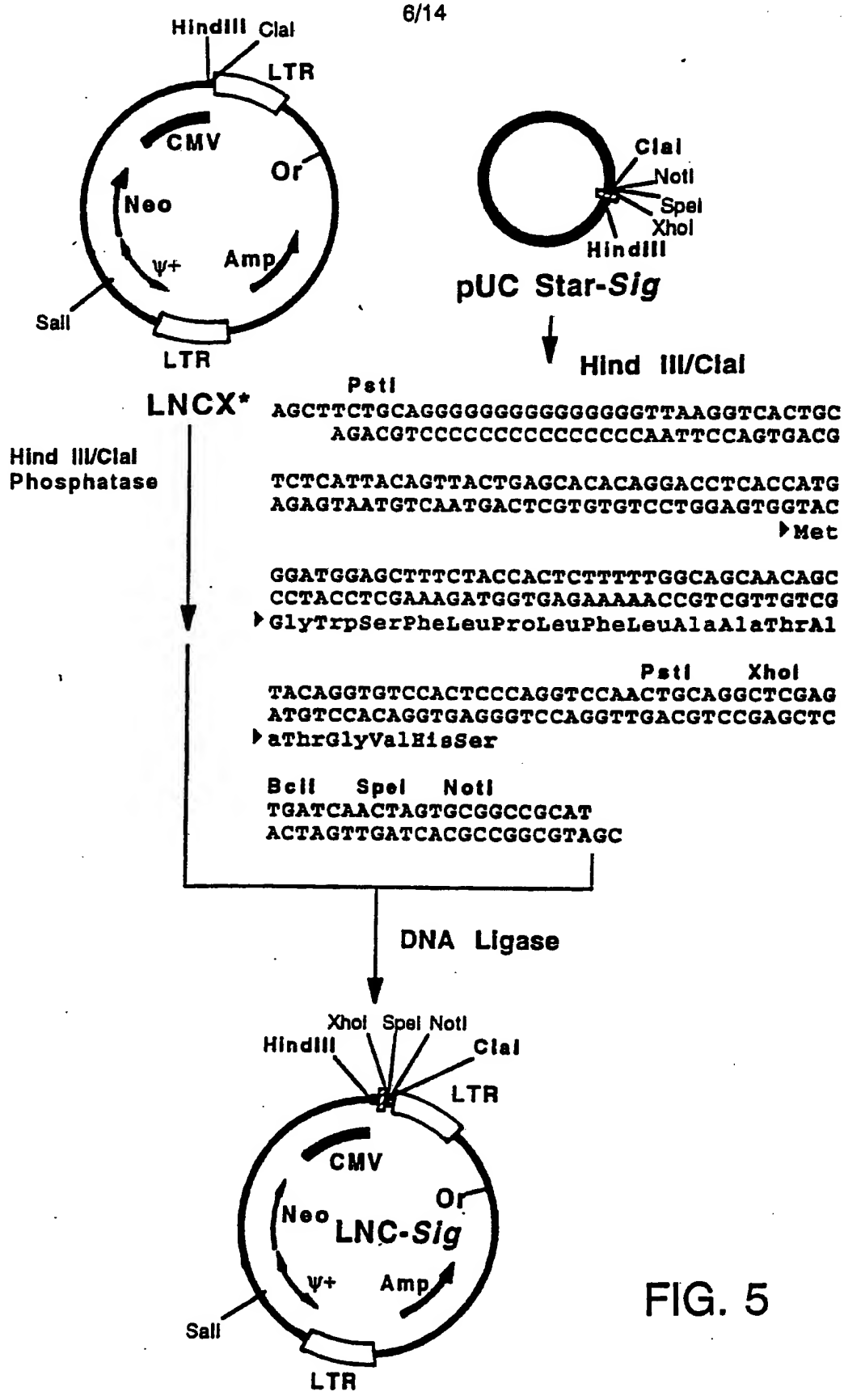
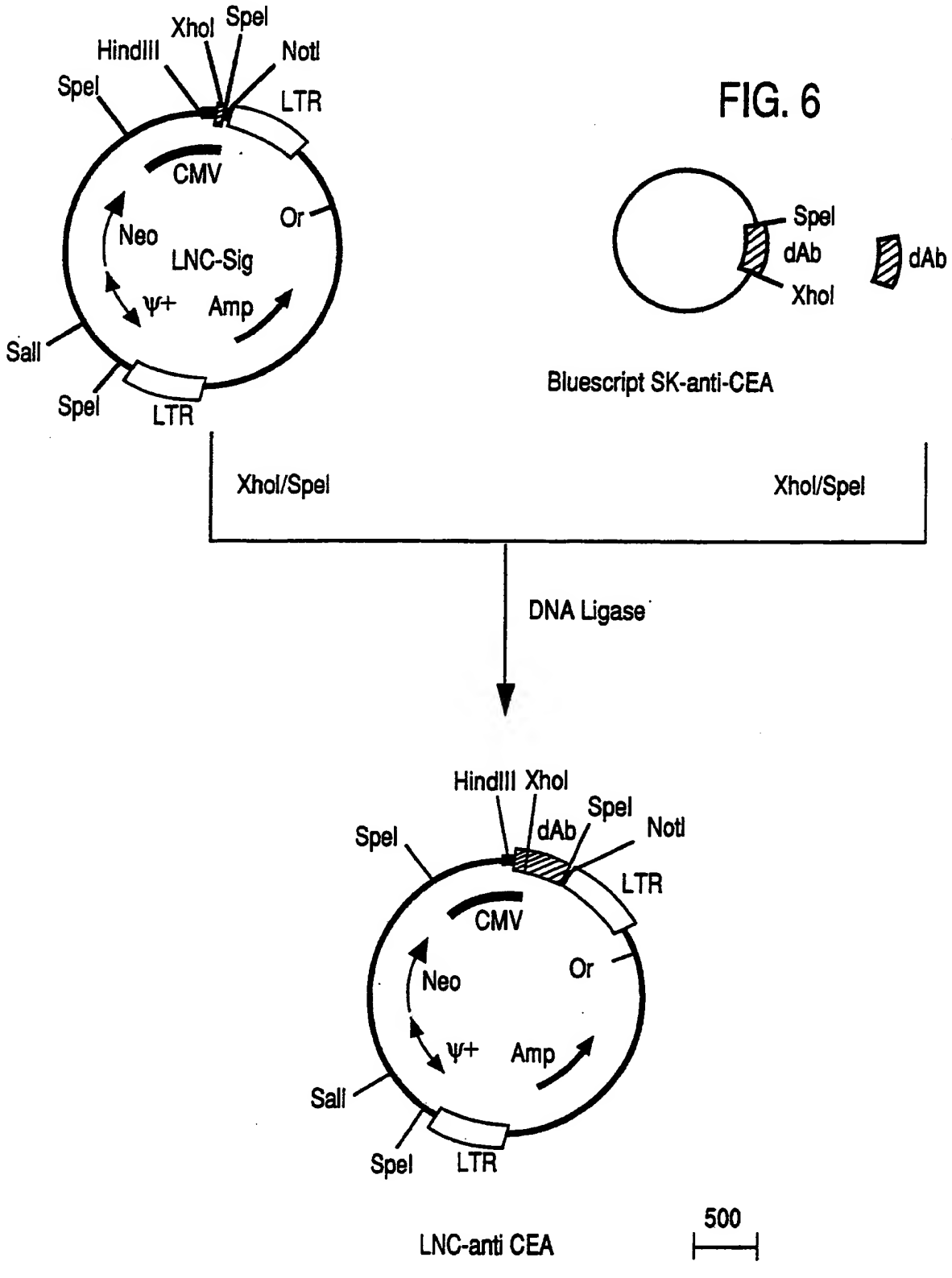

HindIII ClaI
LTR
CMV
Or
Neo
Amp
ψ+
SalI
LTR
ClaI
NotI
SpeI
XhoI
HindIII
pUC Star-Sig
Hind III/ClaI
LNCX*
PstI
AGCTTCTGCAGGGGGGGGGGGGGGGTTAAGGTCACTGC
AGACGTCCCCCCCCCCCCCCCAATTCCAGTGACG
Hind III/ClaI
Phosphatase
TCTCATTACAGTTACTGAGCACACAGGACCTCACCATG
AGAGTAATGTCAATGACTCGTGTGTCCTGGAGTGGTAC
Met
GGATGGAGCTTTCTACCACTCTTTTTGGCAGCAACAGC
CCTACCTCGAAAGATGGTGAGAAAAACCGTCGTTGTCG
GlyTrpSerPheLeuProLeuPheLeuAlaAlaThrAl
PstI XhoI
TACAGGTGTCCACTCCCAGGTCCAACTGCAGGCTCGAG
ATGTCCACAGGTGAGGGTCCAGGTTGACGTCCGAGCTC
aThrGlyValHisSer
BclI SpeI NotI
TGATCAACTAGTGCGGCCGCAT
ACTAGTTGATCACGCCGGCGTAGC
DNA Ligase
XhoI SpeI NotI
HindIII
ClaI
LTR
CMV
Or
Neo
LNC-Sig
ψ+
Amp
SalI
LTR

FIG. 5

FIG. 6


HindIII
XhoI
SpeI
NotI
SpeI
LTR
CMV
Or
Neo
LNC-Sig
ψ+
Amp
SalI
SpeI
LTR
SpeI
dAb
XhoI
dAb
Bluescript SK-anti-CEA
XhoI/SpeI
XhoI/SpeI
DNA Ligase
HindIII
XhoI
dAb
SpeI
NotI
SpeI
LTR
CMV
Or
Neo
ψ+
Amp
SalI
SpeI
LTR
LNC-anti CEA
500

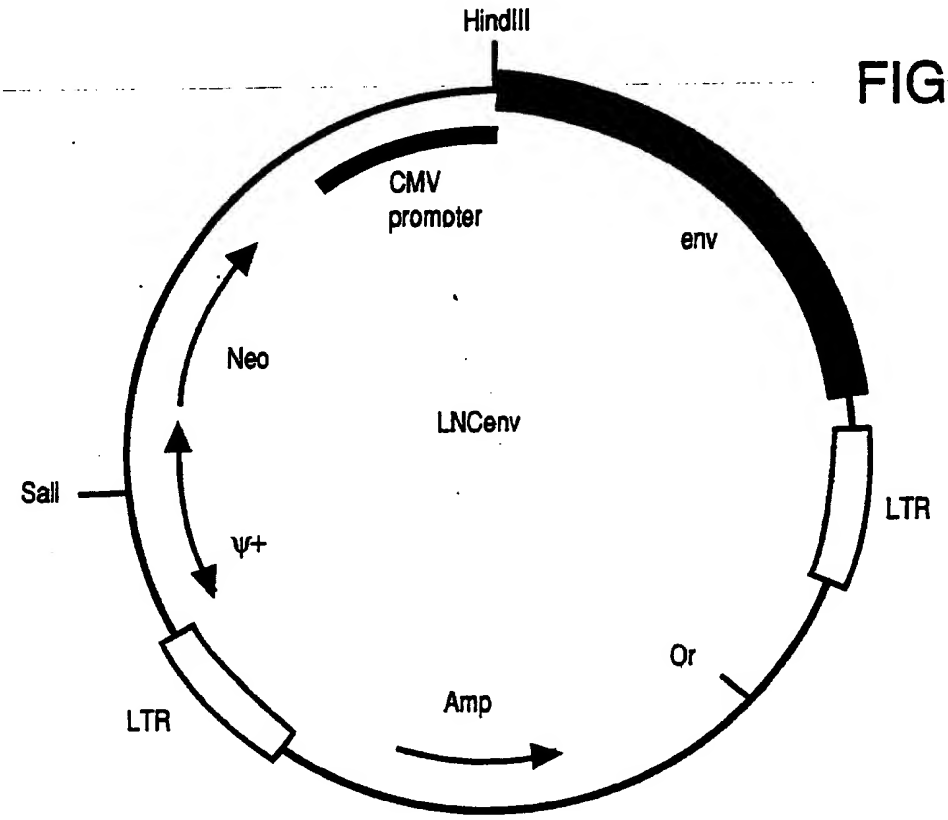

HindIII
CMV
promoter
env
Neo
LNCenv
SalI
LTR
ψ+
Or
Amp
LTR

FIG. 7A

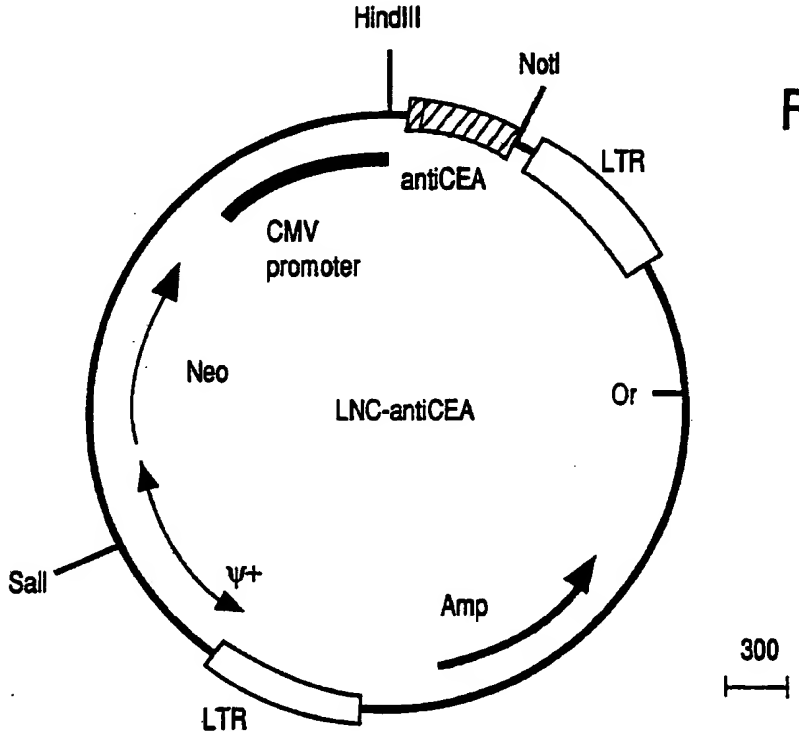

HindIII
NotI
LTR
antiCEA
CMV
promoter
Neo
LNC-antiCEA
Or
SalI
ψ+
Amp
LTR
300

FIG. 7B

FIG. 8

Strategy for generating targeted retroviruses

1. Construction of hybrid Ig-env genes in vitro

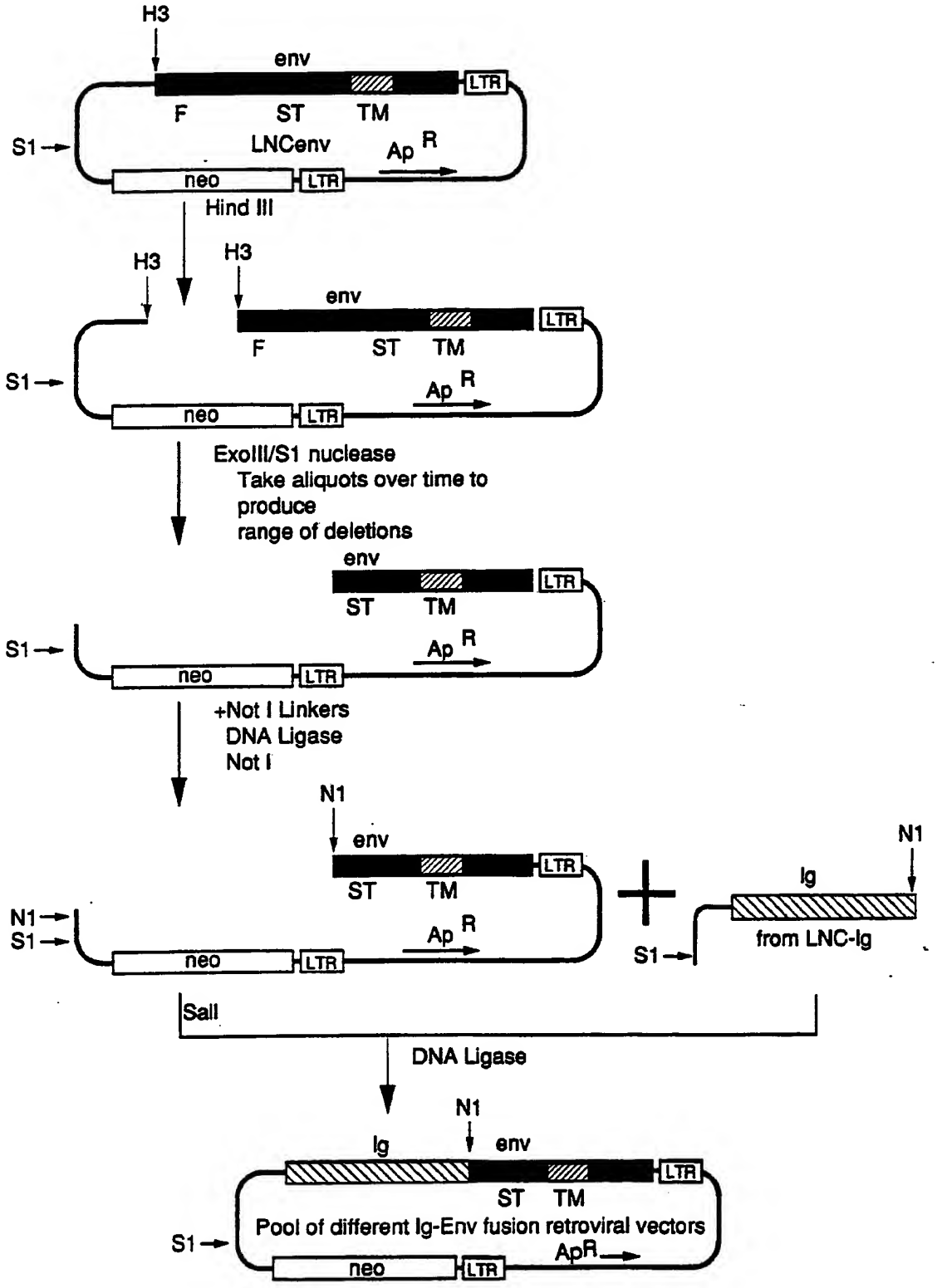


FIG. 9

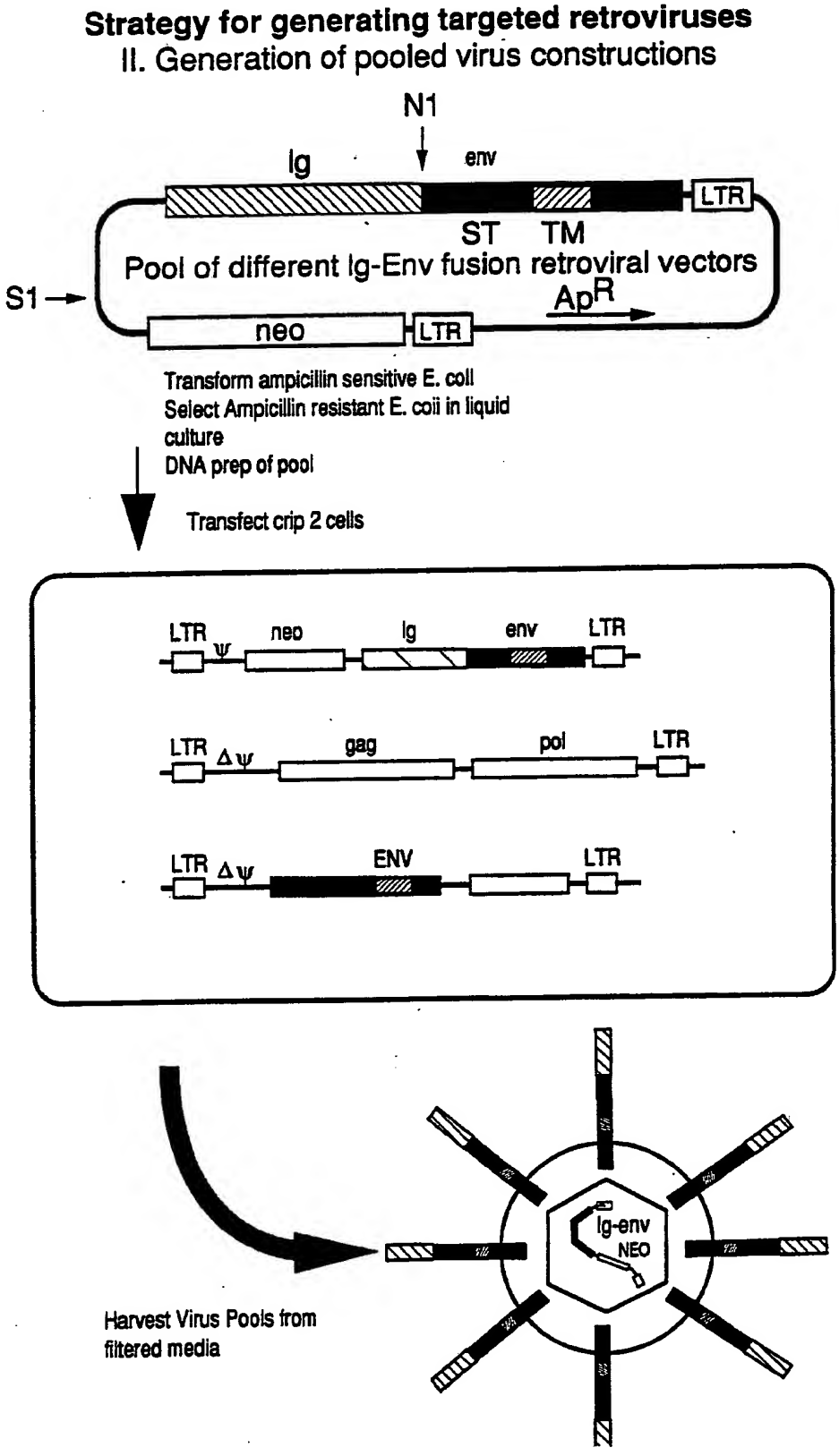
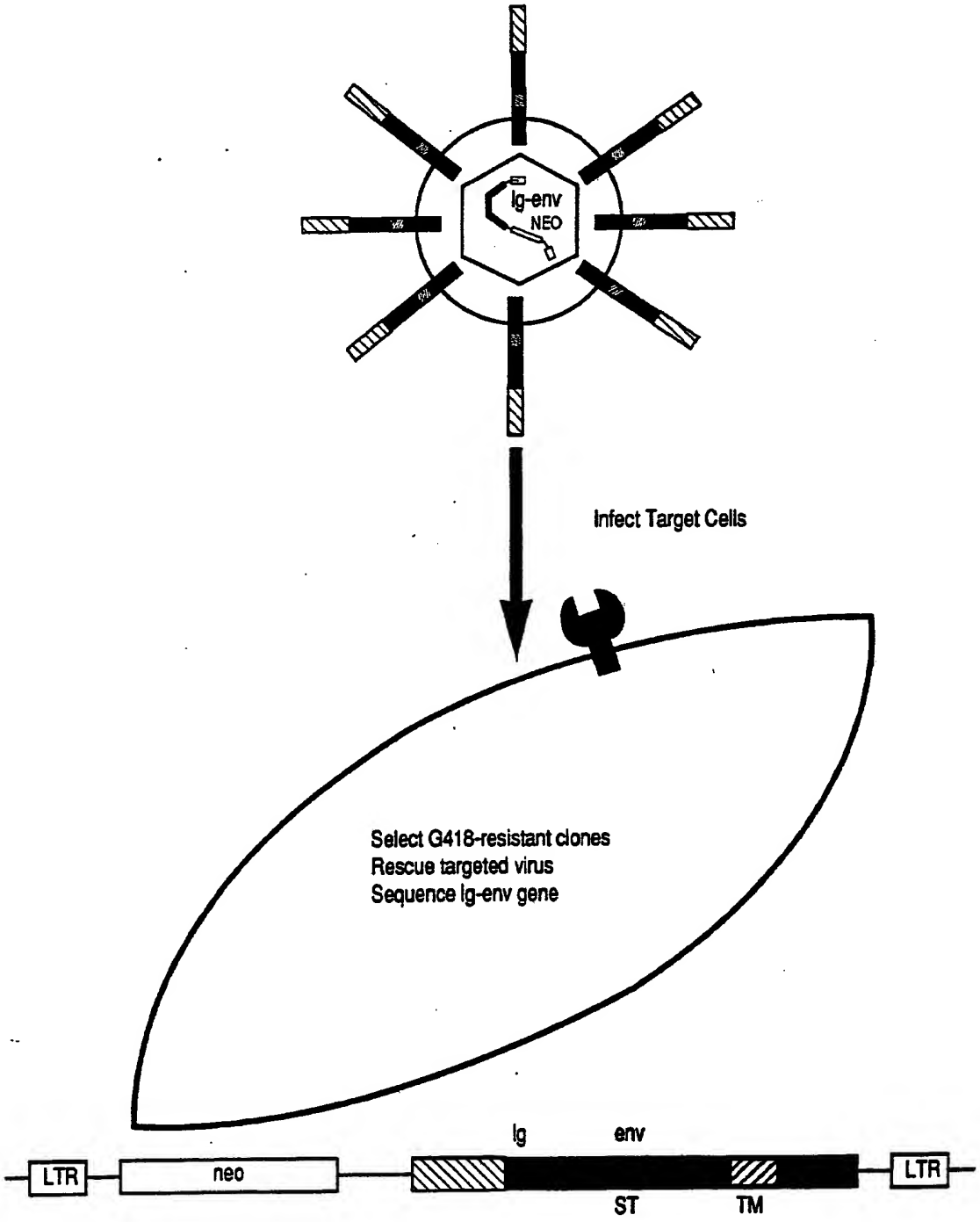

Strategy for generating targeted retroviruses
II. Generation of pooled virus constructions
N1
Ig
env
LTR
ST
TM
Pool of different Ig-Env fusion retroviral vectors
S1
ApR
neo
LTR
Transform ampicillin sensitive E. coli
Select Ampicillin resistant E. coli in liquid culture
DNA prep of pool
Transfect crip 2 cells
LTR
ψ
neo
Ig
env
LTR
LTR
Δψ
gag
pol
LTR
LTR
Δψ
ENV
LTR
Ig-env
NEO
Harvest Virus Pools from filtered media

FIG. 10

Strategy for generating targeted retroviruses

III. Selection and chatacterization of targeted virus



Retrovirus genome encoding targeing Ig-env fusion protein

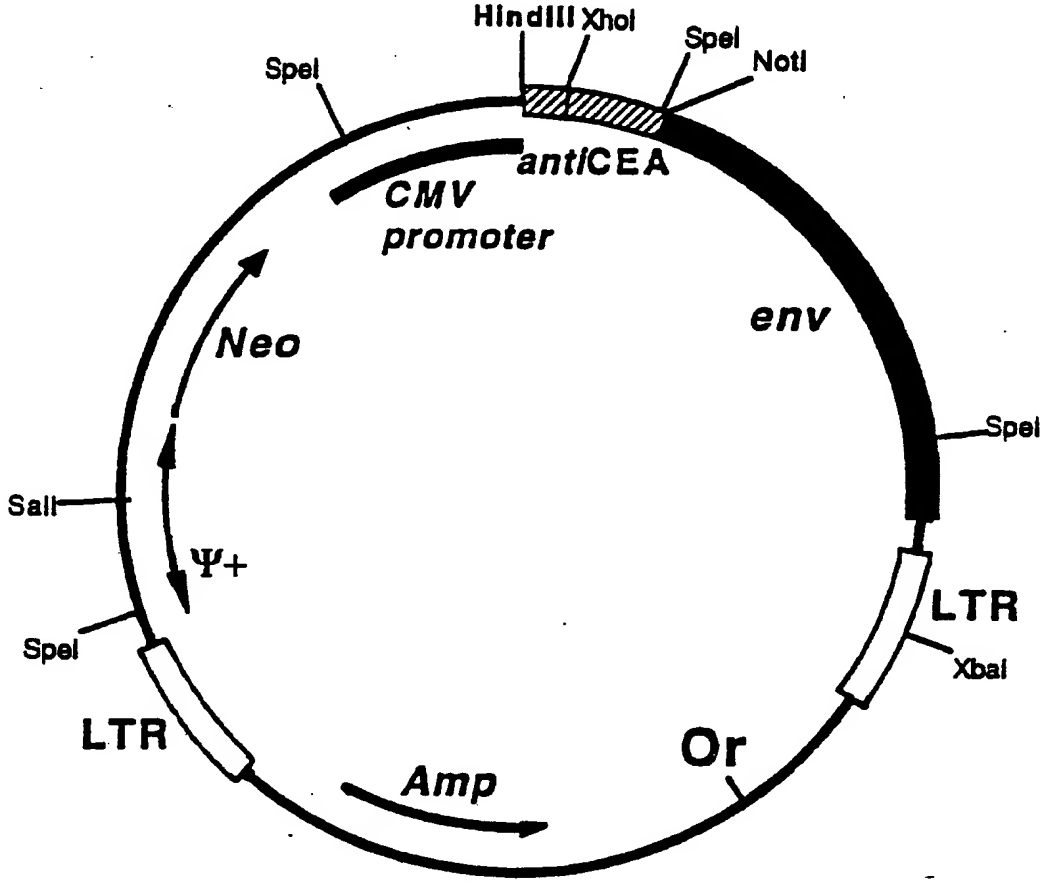

HindIII
XhoI
SpeI
NotI
SpeI
antiCEA
CMV
promoter
env
Neo
SpeI
SalI
Ψ+
LTR
SpeI
XbaI
LTR
Amp
Or

FIG. 11

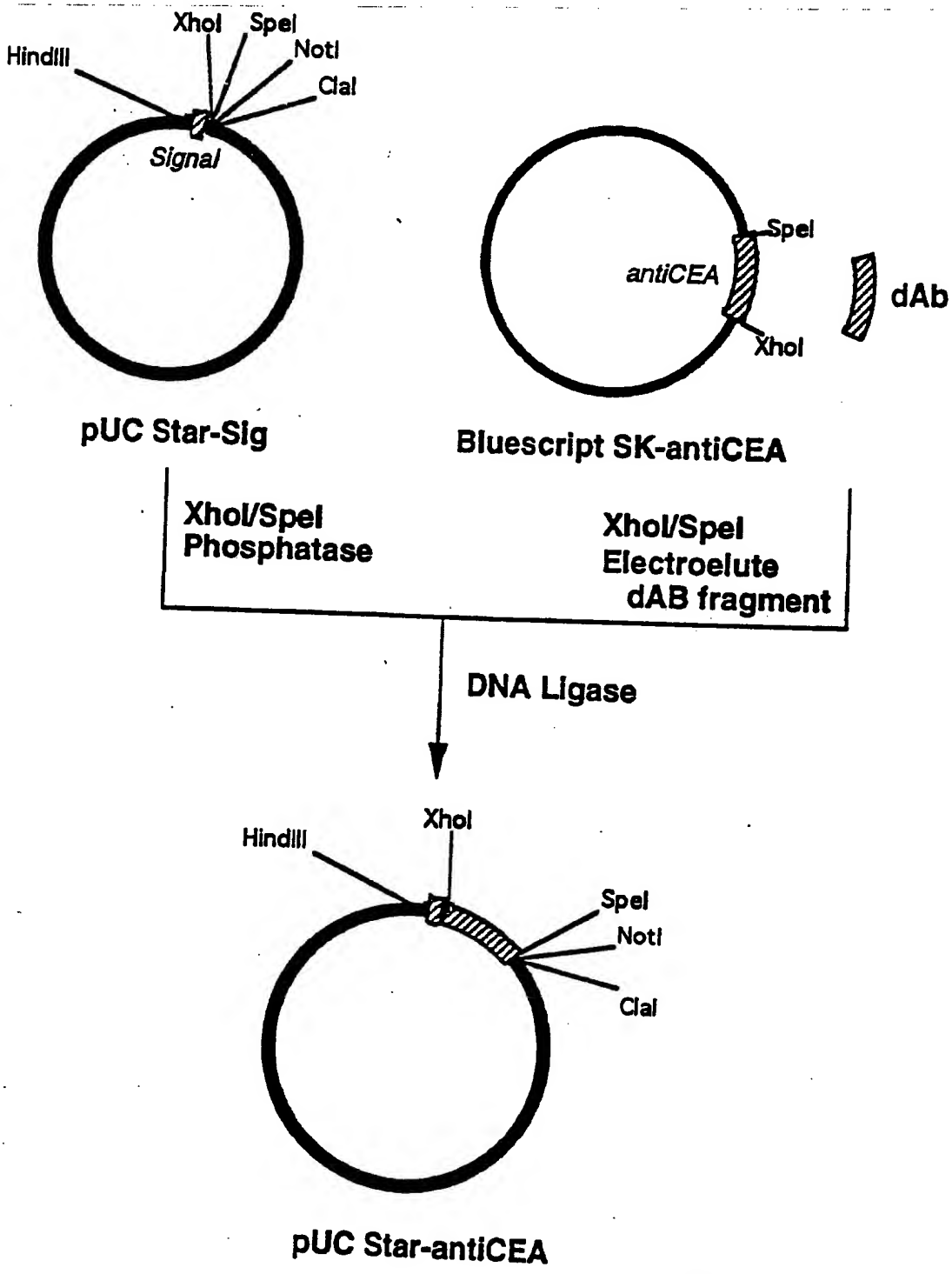

XhoI
SpeI
NotI
ClaI
HindIII
Signal
SpeI
antiCEA
dAb
XhoI
pUC Star-Sig
Bluescript SK-antiCEA
XhoI/SpeI
Phosphatase
XhoI/SpeI
Electroelute
dAB fragment
DNA Ligase
XhoI
HindIII
SpeI
NotI
ClaI
pUC Star-antiCEA
300

FIG. 12

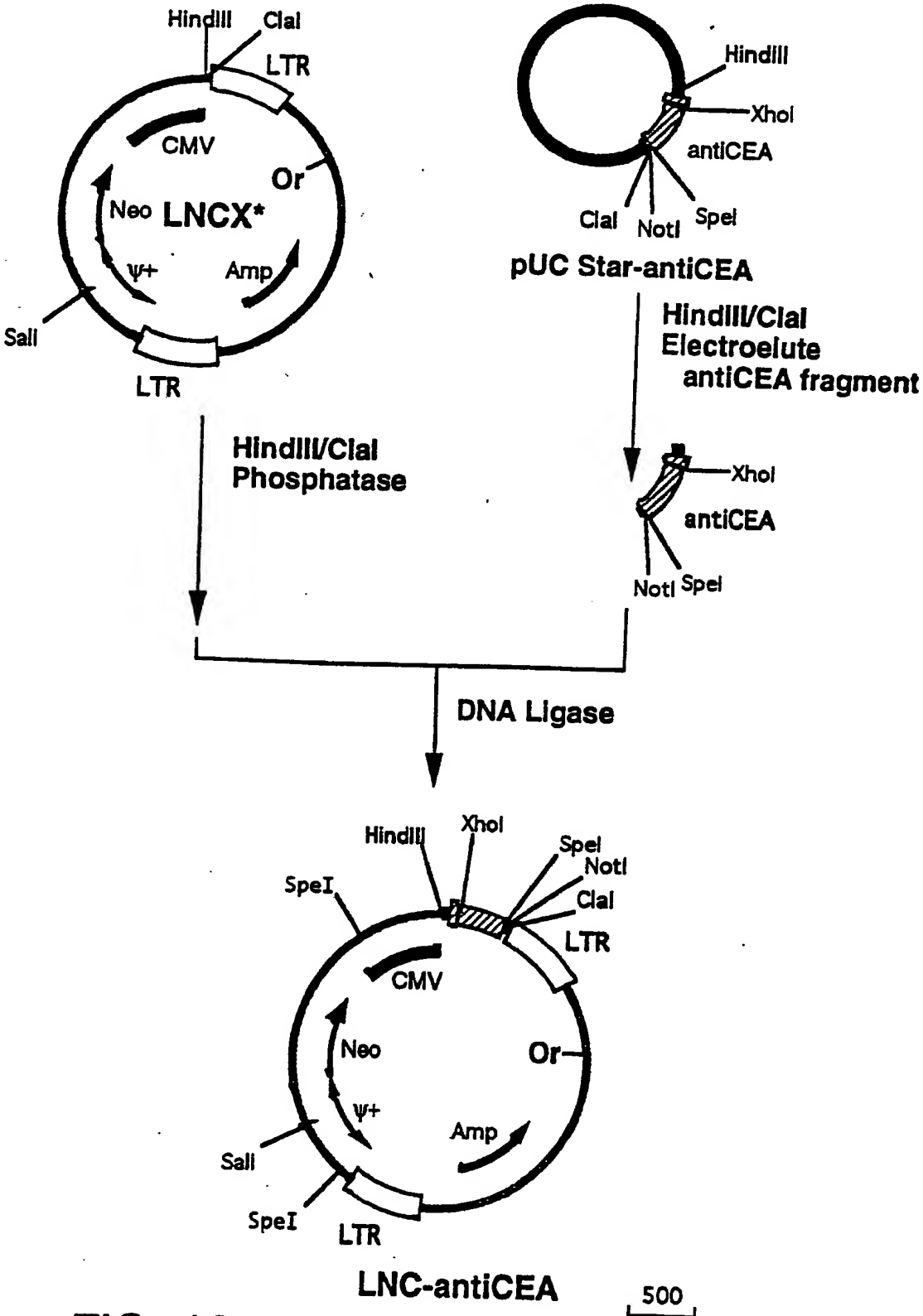

HindIII
ClaI
LTR
CMV
Or
Neo
LNCX*
ψ+
Amp
SalI
LTR
HindIII
XhoI
antiCEA
ClaI
NotI
SpeI
pUC Star-antiCEA
HindIII/ClaI
Electroelute
antiCEA fragment
HindIII/ClaI
Phosphatase
XhoI
antiCEA
NotI
SpeI
DNA Ligase
HindIII
XhoI
SpeI
NotI
ClaI
SpeI
LTR
CMV
Neo
Or
ψ+
Amp
SalI
SpeI
LTR
LNC-antiCEA
500

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02957

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/86; A61K 49/00
US CL :435/69.1, 320.1; 424/9, 93A

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1; 424/9, 93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: CA SEARCH, MEDLINE, BIOSIS PREVIEWS, WORLD PATENTS INDEX; U.S. PATENTS: APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunology Today, Vol. 11, No. 6, issued 1990, S. J. Russell, "Lymphokine Gene Therapy for Cancer," pages 196-200. See page 198, second column, first paragraph.	1-39
Y	Science, Vol. 250, issued 07 December 1990, J. A. T. Young et al, "Efficient Incorporation of Human CD4 Protein into Avian Leukosis Virus Particles," pages 1421-1423. See entire article.	1-39
Y	Nature, Vol. 340, issued 27 July 1989, J. W. Wills, "Retro-secretion of recombinant proteins," pages 323-324. See page 324, last paragraph.	1-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
15 June 1993

Date of mailing of the international search report
18 JUN 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer
JOHNNY F. RAILEY II

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02957

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Vol. 244, issued 16 June 1989, T. Friedmann, "Progress Toward Human Gene Therapy," pages 1275-1281. See pages 1277-1278.	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02957

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Form PCT/ISA/206 Previously Mailed.)

I. Claims 1, 2, 4-11, 14, 15, 17-28, 30-37, drawn to methods of expression of nucleic acids, viruses, methods of delivery of nucleic acids wherein the virus is Retroviridae.

II. Claims 1-3, 6-11, 14-16, 19-29 and 32-37, drawn to methods of expression of nucleic acids, viruses, methods of delivery of nucleic acids wherein the virus is Herpesviridae.

III. Claims 12, 13, 38 and 39, drawn to in vivo therapies for expressing nucleic acid or delivery of nucleic acids.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.